THE REGULATION OF EXPRESSION OF LOW MOLECULAR WEIGHT RNA SPECIES IN AMPHIBIAN OOCYTES

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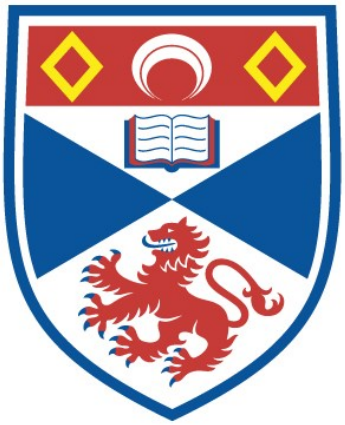

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THE REGULATION OF EXPRESSION OF LOW MOLECULAR WEIGHT RNA SPECIES IN AMPHIBIAN OOCYTES

BY

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DEPARTMENT OF ZOOLOGY

UNIVERSITY OF ST. ANDREWS

OCTOBER 1986

DECLARATION

I hereby declare that this thesis is of my own composition and that the experimental work was performed by me alone apart from a proportion of that reported in Section 1 which was undertaken and published jointly with Dr. P-M. Kloetzel and Dr. J. Sommerville in Biochim. Biophys. Acta, 740 347-354 (1983), R.M. Johnson and Dr. J Sommerville in Exp. Cell Res., 153 299-307 (1984) and Eur. J. Biochem. 144,503-508 (1984).

None of the material in this thesis has been submitted for any other degree.

I was admitted to the faculty of Science of the University of St. Andrews under ordinance No. 12 on October 1981 and as a candidate for the degree of Ph.D. on October 1982.

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

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ABSTRACT

During early oogenesis in Xenopus laevis about half of the 5S ribosomal RNA and most of the transfer RNA produced is stored in a ribonucleoprotein (RNP) complex that sediments at 42S. The other half of the 5S RNA produced is stored in a separate 7S RNP particle. As ribosome production gets underway in mid-oogenesis both the 42S and 7S particles disappear, the 5S rRNA being incorporated into the ribosomes, the tRNA being released as a slower sedimenting particle.

Heterogeneity in the composition of the 42S particle was observed with respect to both protein and RNA components. The proteins of the 42S particle, Mr 48000 (P48) and Mr 43000 (P43) appear to be cleaved to smaller proteins. A derivative of P43 of Mr 17000 (P17) possibly becomes a ribosomal protein.

Several observations are consistent with the view that P43 may accompany 5S RNA to the nucleolus before its cleavage product (P17) becomes incorporated into the ribosome.

The chemical and structural relationships of P48, P43 and the protein component of the 7S RNP particle, transcription factor IIIA were studied and it was established that they are the products of three different genes. The 42S particle proteins have also been shown to have a binding affinity for 5S RNA genes (P48), tRNA genes (P43) or ribosomal genes (P43). In vivo inhibition of 5S RNA transcription by an anti-P48 antibody or tRNA transcription by an anti-P43 antibody suggest that these proteins may have a role in the transcription of these genes.

Interactions between P43 and tRNA genes were analysed in most detail and indicating a specific interaction between these two components. Th results taken as a whole suggest a central role for 42S particle proteins in particular P43, in co-ordinating the formation of the protein translational machinary.

ABBREVIATIONS

A	Absorption at 254nm
bp	Base pair
cRNA	Complementary RNA
Ci	Curie
cpm	Counts per minute
dATP	Deoxyribo-adenosine triphosphate
dCTP	Deoxyribo-cytosine triphosphate
dGTP	Deoxyribo-guanosine triphosphate
dTTP	Deoxyribo-thymidine triphosphate
DEAE	Diethylaminoethyl
DNase1	Deoxyribonuclease 1
EDTA	Ethylenediamime tetraacetic acid
GTP	Ribo-guanosine triphosphate.
Kb	Kilobase pair
kD	Kilodalton
NP40	Nonidet P-40
PMSF	Phenylmethyl sulphonylfluoride
RIA	Radioimmunoassy
RNP	Ribonucleoprotein
SDS	Sodium dodecyl sulphate

GENERAL INTRODUCTION

In recent years many advances have been achieved in elucidating the mechanism and control of eukaryotic gene expression. Recombinant DNA technology and the exploitation of cell-free and injected living systems have assisted in defining the processes involved. It is now evident that there are many opportuntities between the initiation of transcription and utilization of RNA or protein products for control processes to exert an effect on the expression of a gene.

Eukaryotes contain three RNA polymerases, each of which transcribes a subset of genes (1). RNA polymerase 1 is resistant to the fungal toxin α-amanitin. It transcribes ribosomal RNA (rRNA) genes which are organized as clusters of tandem repeats within a nucleolus (2). Each repeating unit is transcribed into a single precursor RNA molecule that is processed to give the mature rRNA species of 18S, 5.8S and 28S.

RNA polymerase II is specifically inhibited by low levels of α-amanitin. This polymerase transcribes protein-coding genes to produce an mRNA molecule which becomes capped, polyadenylated and spliced to remove intervening sequences (3,4). Also transcribed by RNA polymerase II are the 'U' species of small RNAs (4).

RNA polymerase III is inhibited only by high levels of α-amanitin. This polymerase is responsible for the synthesis of 5S rRNA, tRNA and other small nuclear and cytoplasmic RNAs (K,L,M,4.5S RNA) (5,6). This enzyme also transcribes two small RNAs encoded by the adenovirus 2 genome (VAI and VAII) (7) and two small RNAs

transcribed by Epstein-Barr virus (EBERI and EBERII) (8).

The polymerases of eukaryotes, unlike the prokaryotic polymerase cannot recognize directly DNA sequences that act as promotors of transcription. Additional (protein) factors are required. The sequences involved in the control of transcription have been deduced by studying deletion, insertion, substitution and point mutation (8,10-22). Promotors of related genes may contain conserved sequences. For RNA polymerase I, consensus sequences are species specific (9) with invariant nucleotides in higher eukaryotes at position -16(G) and -1(T) which influence transcription considerably (9). A TATA box and homologies to 'CCAAT' or 'GGGCCG' sequences are found in the 5' flanking sequences of most RNA polymerase II genes. RNA polymerase III transcribed genes show homologies within the internal promotor of these genes (23) and the regions containing the internal promotor are interchangeable (24) between genes transcribed by this polymerase.

Crude extracts prepared from eukaryotic cells contain the essential factors required to impart specificity to transcription (1,6,25-30), and allow in vitro initiation at the same start sites as are used in vivo. In the case of RNA polymerase I these factors confer species specificity (25,26). Some of these factors are required for all promotors transcribed by the RNA polymerase (9,30-34). Specific factors required by different genes bind DNA sequences within the promotor regions. Protection of DNA from digestion by deoxyribonuclease after protein binding (35), together with base modifications, have identified the sequences involved and the contact points between factor and DNA. In the case of SpI, a

factor isolated from cultured human cells, the protein binds to a 21bp repeat upstream from the promotor of early transcription in SV40 (36). Factors A and B isolated from Drosphophila are required for RNA polymerase II transcription of certain genes in this organism. Factor B binds to a 65bp region surrounding the start point of transcription, including the TATA box (37). Factor A binds a 55bp region upstream of the TATA box of a gene expressing a protein in response to heat shock, hsp 70 gene (38). Sequences involved in binding and the contact points of TFIIIA to the 5S RNA gene promotor has been well characterized (39,40), as well as factor τ binding to $tRNA^{Glu}_{3}$ gene promotor of yeast (41).

The full complement of transcription factors required by an eukaryotic RNA polymerase has been ascertained by ion-exchange chromatography of crude cell extracts. RNA polymerase I requires as many as four fractions after ion-exchange chromatography (42), RNA polymerase II requires three fractions after ion-exchange chromatography (31,32,38,43) as does RNA polymerase III (30,34). The binding order, complex stability and other requirements have been analyzed in some detail for 5S RNA genes (44).

All three different types of RNA polymerase are involved in the formation of ribosomes. Normally there is co-ordinated activity of genes transcribing rRNA, 5S RNA and ribosomal proteins to ensure that equal amounts of each component are produced. However, in oocytes these different transcriptional activities are uncoupled, serving to compensate for the vastly different number of genes available (Table 1). Thus the formation of 5S RNA (and tRNA) (45-48) and mRNA encoding ribosomal proteins (49), are activities that occur early in

TABLE 1

NUMBER OF GENES PRESENT IN OOCYTES
AND SOMATIC CELLS OF *XENOPUS LAEVIS*

	NUMBER OF GENES	
TYPE OF GENES	OOCYTES	SOMATIC CELLS
28S + 5.8S + 18S	2,000,000	1,000
5S RNA	96,000	48,000
tRNA (total of 40 kinds)	32,000	16,000

oogenesis. In early (previtellogenic) oocytes up to 80% of the total RNA consists of 5S RNA and tRNA (46,50,51) and 10-20% of the 10-16S polyadenylated RNA encodes ribosomal protein (52). Only during vitellogensis do vast numbers of rRNA genes become fully active (53) so that by the end of oogenesis approximately equimolar amounts (10^{12} molecules/ oocyte) of each ribosomal component have been produced. Of course production from the non reiterated r-protein genes are, in effect, amplified through the additional step of translation.

The 5S RNA and tRNA molecules stored during previtellogensis are stabilized through their association with a set of three abundant proteins to form ribonucleoprotein (RNP) particles. Both 5S RNA and tRNA molecules interact with two proteins of molecular weight 48,000 (P48) and 43,000 (P43) to form an RNP particle that sediments at 42S (46,54), whereas 5S RNA alone interacts with a protein of molecular weight 40,000 (P40) to form an RNP particle that sediments at 7S (55).

In this thesis I have examined the potential involvement of these three proteins in co-ordinating the activities of 5S RNA, tRNA and ribosomal RNA and their potential involovement in ribosome formation/ structure, roles which are crucial in forming the protein synthetic machinery to be used by the developing organism.

INTRODUCTION

Previtellogenic oocytes contain large amounts (10^{12} molecules) of 5S RNA of which only a small amount (less than 10% (46,47)) is complexed in ribosomes. This is because many of the other ribosomal components are not present in sufficient quantities at this stage of oogenesis (50,56). However in somatic cells, this situation does not arise and most of the 5S RNA is found associated with ribosomes. In oocytes, the non-ribosome-bound 5S RNA is not found as free RNA, but half* is recovered in a 7S particle in which the 5S RNA is associated with a single protein (55,57) the other half being recovered in a high molecular weight complex sedimenting at 42S which also contains tRNA (46,54) in addition to protein components.

1:1 7S particles

Although 7S particles (55) were discovered several years after 42S particles (46,54), much more is known about the components of the smaller particle.

The biochemical and physical properties of the 7S particle have been analysed in detail. The particles contain approximately equal amounts of 5S RNA and protein (55). The particle may be purified by centrifugation through sucrose, by chromatography on ion-exchange resins, or by preparative electrophoresis (55). The protein moiety may be purified from these particles by dissociation of the RNA by ionic detergents or by ribonuclease treatment which leaves the protein in a precipitated state (58). A single protein is released from the 7S particles which has a molecular weight of 32000 in the

fish Tinca tinca (57), and 40000 in the amphibian Xenopus leavis (55) as measured by SDS gel electrophoresis. More recently, the gene for X.laevis 7S RNP protein has been cloned and the amino acid composition and primary sequence has been derived from the cloned DNA (59). The interaction of 5S RNA with the 7S particle protein has been probed using a variety of ribonucleases, which together with the ability of the protein to recognise heterologous eukaryotic 5S RNA, suggests that it can recognise a generalised eukaryotic 5S RNA structure (60,61).

A role as a storage protein for the 7S particle protein is only one function of this protein. It has been shown that this protein stimulates transcription of 5S RNA in an in vitro system containing cloned 5S RNA genes and RNA polymerase III (39,62,63). As a consequence, this protein is known as transcription factor A for RNA polymerase III or TFIIIA for short.

1:2 42S RNP particle

42S RNP particles are purified by sucrose density centrifugation (46,54). They contain both RNA and protein which may be seperated by ionic detergents, indicating that they are held together by weak non-covalent bonds (64).

Electrophoretic analysis of the proteins in this particle from X.laevis T.tinca and Triturus cristatus reveal two major proteins of 45 KDa (P45) and 39 KDa (P39) in T.cristatus, 48 KDa (P48) and 43 KDa (P43) in X.laevis, and 50 KDa and 40 KDa in T.tinca (64,65,57). Analysis of the molar ratios of tRNA and 5S RNA in the particle by

gel electrophoresis and gel filtration through Sephadex G-100 revealed that the ratio was 3 for tRNA/5S RNA, for X.laevis, T.tinca and T.cristatus (46,54,65). The molar ratio of the protein components was found by electrophoresis to be 2 molecules of P48 to 1 molecule of P43 in X.laevis, and the same ratio for the equivalent proteins in other species (64,66).

The 42S particle may be dissociated using higher salt concentrations during isolation, with a decrease in the 42S peaks and a concomitant increase in peaks of 25S and 15S with an increasing salt concentration. All these peaks contain the same RNA and protein components as the 42S peaks. These slower sedimenting particles represent dimers and monomers of the components of the 42S particle, which is a tetramer of the 15S monomer (51,57,64,66).

No role other than the storage of 5S RNA and tRNA to prevent degradation of these RNAs has been proposed for this particle, although its ability to affect transcription of 5S RNA genes was tested but yielded no positive result (62).

1:3 Ribosomes

Ribosomes from eukaryotic cells are composed of four different RNA molecules (with sedimentation values of 5S, 5.8S, 17-18S and 25-28S), and about 70-80 different proteins. Therefore, the formation of ribosomes is a very complex process which requires a) the synchronised production of all ribosomal constituents and thus a highly coordinated expression of a large number of genes; b) the assembly of the various components into preribosomal particles in an

ordered fashion, and c) subsequent processing of the preribosomes into mature ribosomal subunits in a very accurate way (67,68).

Regulation of the synthesis of ribosomes occurs both at the level of transcription and at the level of assembly and processing. The latter type of regulation is particularly important in situations where the growth of cells is slowing down or where there is an unbalanced production of the various ribosomal constituents. In cases of an insufficient supply of one or more of the ribosomal constituents, irrespective of which, the excess of the other ribosomal constituents is eliminated (69,70).

Apparently, ribosomal (precursor) RNA and ribosomal proteins are degraded if they are not promptly assembled and processed into mature ribosomes. Under balanced growth conditions, however, there is little, if any, degradation of any of the ribosomal constituents, at least in so far as this has been examined. Therefore there must be an almost perfectly balanced synthesis of all ribosomal components, most likely by a coordinate control of transcription of the ribosomal genes (46,71). This coordinate synthesis of the ribosomal components in eukaryotes is the more remarkable as three different kinds of RNA polymerases are involved in the transcription of the ribosomal genes. The ribosomal protein genes are transcribed by RNA polymerase II, the genes coding for 17-18S, 5.8S and 25-28S rRNA form a transcriptional unit which is transcribed by RNA polymerase I and 5S rRNA is synthesised separately by RNA polymerase III.

In Xenopus oocytes however, coordinate synthesis of ribosomal components does not take place during early oogenesis (53). Instead, 5S RNA (and tRNA) are synthesised in vast amounts during the previtellogenic stages. This synthesis does not rely on amplification of 5S RNA genes. This is not the case for the 18S, 5.8S and 28S rRNA genes. The genes coding for these rRNAs are selectively amplified during early oogenesis (72,73). This results in the oocyte becoming polyploid for these rRNA genes, but remains tetraploid for the rest of the genome. These amplified genes are organised into extrachromosomal nucleoli which are visible in the germinal vesicle in cytological preparations. These nucleoli function independently of the chromosomes (74).

In this section of the thesis I examine the relatedness of the protein components of the 42S RNP and 7S RNP particles, their interaction with 5S RNA and tRNA, and their storage, distribution and utilisation during oogenesis The key events in ribosome synthesis/assembly are largely unknown in eukaryotes (75), but due to the vast amount of ribosome synthesis that takes place during oogenesis and the uncoupled production of different ribosomal components (58,49), oocytes offer a system in which ribosome assembly may be studied. In this section I also examine the potential involvement of the 42S particle proteins in ribosome assembly.

MATERIALS AND METHODS

1:4 Fractionation of homogenates of *Xenopus* Oocytes

For previtellogenic ovary, female *Xenopus laevis* were reared in our laboratory and used at 3-6 months postmetamorphosis. Mature wild *Xenopus* were obtained from Xenopus Ltd, Redhill, Surrey.

Routine preparation of 42S and 7S particles for preparative and analytical purposes was from previtellogenic ovary, whilst ribosomes were routinely prepared from mature ovary. The procedure is outlined below.

Ovary was dissected from young or mature *Xenopus laevis*. If necessary, oocytes were released from ovarian tissue by stirring for 2 hr at 20°C in the solution OR2 minus Ca^{2+} (82.5mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, 1mM Na_2HPO_4, 5mM HEPES, 0.05% polyvinylpyrrolidone, 3.8mM NaOH pH7.8) (76), plus 0.2% collagenase (Sigma, type IV). Released oocytes or ovaries were washed free of collagenase in three changes of 20 vol OR2 minus Ca^{2+}, then in three changes of 20 vol modified Barths solution (88mM NaCl, 1mM KCl, 2.4mM $NaHCO_3$, 0.82mM $MgSO_4.7\ H_2O$, 0.33mM $Ca(NO_3)_2.4\ H_2O$, 0.41mM $CaCl_2.6\ H_2O$, 7.5mM Tris-HCl pH 7.6 plus 5 units of pencillin, streptomycin and kanamycin/ml) (53) and grouped into developmental stages as described by Dumont (77). Ovaries or staged oocytes were washed in modified Barths solution, and, if required labelled with 0.4mCi/ml ^{3}H-uridine (27Ci/mMol), for 18hrs at 20°C or 0.15mCi/ml ^{35}S-cysteine (1000Ci/mMol), 0.15mCi/ml. ^{35}S-methionine (1000Ci/mmol), 10uCi/ml ^{14}C-amino acids (50Ci/mmol) or ^{32}P-phosphate (500uCi/ml) (all Amersham international), for four days

at 20°C in modified Barths solution. Homogenization was in buffer A (50mM NaCl, 10mM Tris-HCl, pH 7.4, 5mM $MgCl_2$ and 5mM 2-mercaptoethanol) containing 8.5% (w/v)) sucrose as described previously (78). The homogenate was clarified by centrifugation at 1000xg for 30 mins at 2°C or pellets were collected by differential centrifugation, using a Sorvall HB-4 rotor, at 100xg for 5 min (Pellet 1), 3000xg for 10 min (Pellet 2) and 10000xg for 10 min (Pellet 3). Each of the pellets was raised in 2ml of buffer A and recentrifuged as before. The 10000xg supernatants were layered on 15-40% sucrose gradients made up in buffer A. After centrifugation at 21500 rpm for 17 hr at 2°C in an MSE 6x14 ml Ti rotor, or 23000 rpm for 18 hr at 2°C in an MSE 6x35 ml Ti rotor, fractions containing 80S ribosomes, 42S RNP, or 7S RNP particles were collected. All sub-cellular fractions were precipitated with 2 vol ethanol and stored at -20°C overnight or frozen without precipitation at -20° C until required.

1:5 Preparation of liver cell ribosomes

Livers removed from young female Xenopus were diced followed by homogenization in buffer A plus 0.02mM PMSF. The homogenate was spun at 10000xg for 20mins. The supernatant was removed and spun again at 100000xg for 3hrs to pellet ribosomes. The pellet was resuspended in buffer A and layered on to a 15-40% (w/v) sucrose gradient in buffer A and spun at 23000 rpm for 18hr at 2°C in an MSE 6x35ml Ti rotor. The 80S peak was collected and precipitated with 2 volumes of ethanol at -20°C.

1:6 EDTA and salt dissociation of 42S ribonucleoprotein particles and ribosomes

About 2 A_{254} units of 42S ribonucleoprotein particles and ribosomes collected from sucrose gradients were pelleted by centrifugation at 110000xg for 6 hr at 2°C. For EDTA dissociation, the pellet was resuspended in buffer B (30mM NaCl, 10mM Tris-HCl, pH 7.4, 5mM EDTA, 5mM 2 mercaptoethanol) and centrifuged at 13400xg for 2 min to remove insoluble material. The supernatant was layered on 15-40% (w/v) sucrose gradients made up in buffer B and centrifuged in an MSE 6x14 ml Ti rotor at 36000 rpm for 42 hr at 2°C. Peak fractions were collected, precipitated overnight with 2 vol ethanol at -20° C and analysed for protein and RNA constituents by electrophoresis.

For salt dissociation, isolated 42S ribonucleoprotein particles or ribosomes were dialyzed against buffer C (0.5M NaCl, 10mM Tris-HCl, pH 7.4, 2mM $MgCl_2$, 5mM 2 mercaptoethanol) and layered on 15-50% (w/v) sucrose gradient made up in this same buffer. After centrifugation at 38000 rpm in an MSE 6x14ml Ti rotor for 20 hr at 2° C (42S particles), or 16000 rpm in a 6x35 ml Ti rotor for 16 hrs at 20°C (ribosomes), the peak fractions were retained for further analysis.

1:7 Preparation of nucleoli from Xenopus ovary.

The ovary used was taken from a female X.laevis containing mainly stage 2 oocytes. The ovary was homogenised in 5ml of buffer A plus 8.6% sucrose. The homogenate was layered over a 15% sucrose in

buffer A and spun in Sorvall HB-4 rotor at 1000 rpm for 10 mins. A large white pellet was obtained which contained nucleoli (as judged by phase contrast microscopy). The pellet was resuspended in buffer A plus 8,6% sucrose and layered over a 5ml, 40% sucrose plus 3ml 75% sucrose step gradient in buffer A. This was spun in a Sorvall HB-4 rotor at 10000 rpm for 20 mins. A crude preparation of Nucleoli was obtained from the material taken off the 40%/75% sucrose interface. This material was precipitated with 2 volumes of ethanol at -20°C.

1:8 Protein and RNA polyacrylamide gel electrophoresis.

For protein analysis and preparation, SDS-polyacrylamide gel electrophoresis was used according to the modified method of Laemmli (66,80). Proteins were applied to vertical slabs of 12% polyacrylamide, 180x200x1 mm, and run at 200 V for 20 hr.

For RNA analysis and preparation, the denaturing system of Loening (81) was used with 7.5% polyacrylamide, 90x3 mm diam. tube gels or 3-15% or 10% polyacrylamide slab gels. Tube gels were run at 18 mA for 2.5 hr, slab gels at 180 V for 4.5 hr.

Protein gels were stained with either Coomassie brilliant blue (66) or silver salts (82). RNA tube gels were scanned at 254nm using a Joyce-Lobel scanner and/or silver stained. RNA slab gels were stained with acridine orange (15ug/ml).

Protein and RNA were recovered from preparative gels (66) by staining a strip of the preparative track to locate the protein or RNA band of interest then removing the remainder of the band from the preparative track and eluting with an elution buffer for 16-24hrs.

Elution buffer for proteins was 0.1M NaCl, 10mM Tris-HCl, pH7.4, 2mM $MgCl_2$, 5mM 2-mercaptoethanol and 1% SDS. For RNA elutions the buffer was 0.3M Sodium acetate, pH7.4, 1% SDS.

Two-dimensional electrophoresis of ribosomal proteins was carried out as described (52). Breifly ribosomal proteins were extracted in 67% acetic acid, dialyzed against 1% acetic acid then lyophilized. The first dimension separation was essentially as described by Gorenstein and Warner (202), in 2.5 x 120mm tube gels. The second dimension was carried out on a 15% polyacrylamide slab gel as described above.

Iodination of RNA fractions using $Na^{125}I$ was carried out by Dr. J. Sommerville as described previously (83).

1:9 Electrotransfer of proteins to nitrocellulose paper.

A piece of nitrocellulose (0.22μm pore size) was cut to cover the area of polyacrylamide gel to be transferred. The nitrocellulose was pre-soaked in transfer buffer (192mM glycine, 50mM Tris, 20% methanol) (84), then placed over the polyacrylamide gel and air bubbles removed that were trapped between filter and gel. The filter and gel, sandwiched between 'Scotch-Brite' pads and holders was placed in a cell containing transfer buffer (filter at the anode electrode). Transfer was carried out at 30V, 0.1A for 16hrs.

1:10 RNA-protein binding studies

1. In solution ^{125}I labelled RNA (0.2-0.4μg) was mixed with excess denatured protein (2-4μg) in 0.5ml buffer D (0.2M NaCl, 10mM

Tris-HCl, pH 7.4, 2mM $MgCl_2$, 5mM 2-mercaptoethanol) plus 1% (w/v) SDS. The SDS was removed by dialysis against buffer D over a period of 24 hr to allow gradual renaturation (66). Any RNA-protein complexes formed were fixed with 3.5% neutralised formaldehyde and analysed on CsCl density gradients.

2. Filter hybridization. Nitrocellulose containing transferred protein was treated as described for DNA binding (85). Briefly, the transfers were washed for 1hr in 200ml of a solution containing 4M urea, 50mM NaCl, 2mM EDTA, 0.1mM dithiothreitol, 10mM Tris-HCl, pH 7. After three washes, 20 min each, in standard binding buffer (50mM NaCl, 1mM EDTA, 10mM Tris-HCl, pH 7, 0.02% bovine serum albumin, 0.02% Ficoll (Pharmacia), 0.02% polyvinyl pyrrolidone), the transfers were reacted with 0.1 µg (~$4x10^4$ counts/min) ^{125}I labelled 5S RNA and 200ug cold high Mr RNA in 5ml standard binding buffer for 1hr at 18° C. The transfers were then washed three times, 20 min each, in standard binding buffer and dried for autoradiography.

High Mr RNA used to block non-specific binding was collected as flow through from total RNA extracted from Xenopus ovary (86) applied to a column of Sephadex G-100 (Pharmacia) in 10mM Tris-HCl, pH 7.5, 1mM EDTA. This fraction contains mainly 28S plus 18S RNA with about 2% polyadenylated RNA, but no detectable 5S RNA or tRNA.

1:11 Determination of bouyant density

Formaldehyde-fixed material was analysed on 25-60% (w/v) preformed CsCl gradients containing 10mM potassium phosphate buffer, pH 7.2, 3.6% neutralised formaldehyde and 0.1% NP40 (66,81,87). The

RNA:protein ratio in peak fractions was calculated according to Spirin (88). Molecular composition was based on Mr values for 5S RNA and tRNA of 40000 and 25000 respectively.

1:12 Chemical analysis

For amino acid analysis, electrophorectically purified polypeptides were prepared from RNP particles as described above, dialyzed extensively against distilled water and hydrolyzed in 6N HCl, 0.1M thioglycolic acid at 110°C for 24 hr under an atmosphere of N_2. The residues were analysed using an automated single-column analyser.

For cyanogen bromide cleavage patterns the electrophorectically purified polypeptides were dissolved in 70% (v/v) formic acid and incubated with a 100 molar excess of CNBr at 20°C for 48 hr. The cleavage products were precipitated with 3 vol ethanol at -20°C overnight and the dried pellets were raised in electrophoresis sample buffer and applied to a 20% SDS protein gel as described above. Electrophoresis was at 150 V for 16 hr. Polypeptide fragments were located by staining the gel with silver salts as above.

1:13 Antibodies

Antibodies were raised against electrophorectically purified P48 and P43 by reconstructing RNP complexes as follows.

Isolation of 42S RNP or 7S RNP proteins

Gradient fractions containing 42S RNP and 7S RNP particles were precipitated with 2 volumes of 100% ethanol at -20°C overnight. Precipitates were collected by centrifugation at 8000 rpm (10000xg) for 30 min in a Sorvall HB4 rotor. The pellets were then vacuum dried and raised in SDS sample buffer. The proteins were boiled for 5 min before applying to a 12% polyacrylamide gel as described in Section 1.8. preparative purposes, the wells in the gel were made 40mm wide. The proteins were located by cutting a strip from the side of the preparative track and staining in Coomassie blue. Gel slices containing P48, P43 and P40 were cut out, minced into small pieces, and frozen overnight at -20° C. To the frozen fragments, 1 ml of elution buffer containing 1% SDS, 0.1M NaCl, 2mM $MgCl_2$, 5mM 2-mercaptoethanol, 10mM Tris-HCl, pH 7.4 was added and protein was allowed to diffuse out for 24 hr at 20°C.

Isolation of 5S RNA and tRNA

Precipitated 42S RNP particles were raised in a solution containing 100 µg/ml protease K, 1% sarkosyl, 20mM EDTA, 50mM Tris-HCl, pH 8.4, preincubated at 20°C for 30min. After a 1-2 hr incubation at room temperature, 1/10 volume of 0.3M NaCl was added. This was followed by a phenol/chloroform extraction and ethanol precipitation at -20°C overnight.

The RNA precipitate was spun down, vacuum dried and raised in 1/3 strength Loening RNA electrophoresis buffer. The RNA was applied to a 10% RNA gel as described above, in a 30 x 10 x 1mm preparative

track. The 5S RNA and tRNA were located as described for protein, except the stain used was 5μg/μl ethidium bromide. Elution was the same as for protein, except the elution buffer was 1% SDS, 0.6 M Sodium acetate, pH 7.5.

Raising of antibodies

Antibodies were raised against electrophorectically purified P48 and P43 by reconstructing RNP complexes as described in section 1:10, but using equimolar amounts of protein and tRNA. The reconstituted RNPs were injected in 100-200 μg amounts as multiple emulsions into rabbits (78). Antibodies were raised against P40 by isolating 7S RNP particles from previtellogenic ovary as described above. The 7S RNP peak was purified by recentrifugation through a second sucrose gradient, and this naturally-formed RNP complex was used as antigen. P40 was the only polypeptide seen in stained protein gels using this preparation. In no instance were antibodies detected that reacted with the RNA component.

1:14 Radioimmunostaining and radioimmunoassays

Polypeptides derived from 42S and 7S RNP particles were seperated on 12% polyacrylamide-SDS gels as described above, and electrophorectically transferred to nitrocellulose paper (Schleicher and Schuell, BA 83, 0.2 μm pore size) in 192 mM glycine, 50mM Tris, 20% methanol at pH 8.3 (84). Transfers were made at 30 V, 0.1 A for 16-22 hr. Tracks stained with amido black showed that the polypeptides were transferred in proportion to the amounts loaded. The transferred polypeptides were immunostained as described

previously (89). This involves blocking unbound sites on the nitrocellulose with a 5% bovine serum albumin solution in 0.9% (w/v) NaCl, 10mM Tris-HCl, pH 7.4 (TBS) at 32°C for 45 min. The nitrocellulose filters are then incubated with the antisera at dilutions of 1:20 in the above blocking solution. After incubation with antiserum at 20°C for 2 hr, the transfers were washed in TBS plus 0.05% NP40 for 5min and then one more wash in TBS. The filters were then incubated with ^{125}I protein A, 0.2 μCi per ml of blocking solution described above (>30 mCi/mg, Amersham International) at 20°C for 45 min. Following this incubation, the filter was again washed as above, blot dried between two pieces of tissue, and set up for autoradiography with Kodak X-Omat S X-ray film and intensifying screens at -70°C for 2-6 days.

For the radioimmunoassays, 42S and 7S particles were adjusted to 10μg/ml in 0.1M NaCl, 10mM sodium phosphate, pH 7.0 (PBS). Aliquots of 100ul of this solution were added to the wells of microtitre plates (Linbro, Flow Laboratories Inc.) and incubated at 32°C for 1 hr, then at 4°C overnight. After washing the plates thoroughly in five changes of PBS, free reactive sites were blocked by incubating with diluent (3% (w/v) bovine serum albumin in PBS) for 2 hr at 32°C. Antisera were diluted in the wells to give a series of five-fold dilutions, final volume 100 μl, and incubated at 32°C for 4 hr. The plates were washed with PBS as before, and incubated overnight at 18°C with 100 μl per well of ^{125}I labelled protein-A (2.5×10^6 cpm per ml of diluent). After repeated washing with (at least 6 changes of) PBS, individual wells were cut out and counted to determine the radioactivity bound.

1:15 Assay of transcription inhibition in vivo by antibodies

To prepare IgG from the serum, the following procedure was used. Preswollen DE-52 cellulose was equilibrated in 100mM potassium phosphate buffer, pH 7.2. A column of 20cm height by 1cm diameter was poured and then equilibrated with 15mM potassium phosphate buffer. 2ml of serum was applied to the column and eluted with 15mM potassium phosphate buffer. The eluant was scanned at 280nm and the peak eluting immediately after the void volume was collected and taken as IgG. The concentration of IgG was determined spectrophometrically at 280nm (1.4mg IgG/ml = 1 O.D. unit).

For microinjection, the antibodies were first lyophilized and raised in 44mM NaCl, 0.5mM KCl, 7.5mM Tris-HCl, pH 7.6. The microinjection into germinal vesicles of oocytes has been described (90). Firstly, ovary containing stage 2/3 oocytes was collagenased in OR2 minus Ca^{2+} containing 0.2% collagenase (Sigma) for 60-80 min at 20° C. Released oocytes were washed three times with OR2, then three times with modified Barths solution. Groups of 50 stage 2/3 oocytes were transferred to a petri dish (3 cm diameter) containing a nylon grid (mesh 1 mm) fixed on the bottom of the petri dish. Before injection, the Barths solution was removed to just above the oocytes and the dish was centrifuged at 300xg for 12 min at 20°C. This fixed the oocytes to the grid and brought the germinal vesicle to the surface of the oocyte. The germinal vesicle was then injected with 20nl of injection mixture containing 160µg/ml of plasmid DNA, 5.5mg/ml IgG, 4mCi/ml α^{32}P-GTP (750 Ci/mM New England Nuclear) in 44mM NaCl, 0.5mM KCl, 7.5mM Tris, pH 7.6. The oocytes were covered with Barths solution containing the antibiotics penicillin,

streptomycin and kanamycin, all at 5 U/ml, and incubated for 18 hr at 20°C.

To extract the RNA, the oocytes were collected from the grid, then washed with Barths solution. The oocytes were then lysed in lysis buffer (1% Sarkosyl, 50mM Tris-HCl, pH8.3, 20mM EDTA) containing protease K (Boerinhger) at a concentration of 100µg/ml which had been preincubating in the buffer at 20° C for 30 min. The lysed oocytes were incubated with the protease for 2hr at 20° C. The lysate was adjusted to 0.3M NaCl with 3M NaCl, and the RNA extracted using the phenol/chloroform and precipitated with 2 vol. ethanol at -20°C overnight. The RNA was analysed by gel electrophoresis as described above. Autoradiographs were made after drying the gel down under vacuum, as described above, and exposure in contact with X-ray film (Kodak, X-Omat S) for three days.

1:16 Isolation of nuclei

Nuclei were isolated manually from oocytes into a solution containing 70mM KCl, 5mM $MgCl_2$, 2mM dithiothreitol, 20mM Tris-HCl, pH 7.5 and 2% (w/v) polyvinylpyrrolidine. This solution caused gelling of the nucleoplasm and prevented loss of nuclear contents (91). Batches of 60 nuclei were collected in 70% ethanol and stored at -20° C.

1:17 Immunoprecipitation

Antisera were titrated by solid phase radioimmunoassay using RNP particles as antigens (described above). Preimmune sera from all rabbits were checked routinely for negative reaction by this same technique, and also by immunoblotting as described above.

Immunoprecipitation of RNP particles was carried out using antibodies bound to protein-A Sepharose CL-4B (Pharmacia). The Sepharose beads first were washed and equilibrated in buffer A and then 1.5 ml of antiserum was added to 1 ml packed beads plus 2.5 ml buffer A. After incubation at 20°C for 1hr with constant agitation, the beads were collected by centrifugation, washed five times with 5 ml buffer A and finally added directly to RNP particles contained in sucrose gradient fractions. To each 1 ml fraction containing 40-50mg RNP particles was added 0.15 ml of antibody-bound beads and the mixture was agitated at 20° C for 1hr. These conditions were determined by assaying the kinetics of binding of radiolabelled RNP particles to the antibody-bound beads. The beads were recovered by centrifugation and washed three times as described above. Bound RNP particles were released by the addition of SDS to 0.5% and

RNA was extracted as described above (66).

1:18 Microinjection of labelled 42S and 7S particles

42S and 7S particles were labelled in vivo with ^{14}C or ^{35}S amino acids as described in Section 1:4. Following centrifugation, the 42S and 7S fractions were collected, then concentrated approximately 100-fold by dialysis at 4° C against 20% polyethylene glycol in a solution containing 50mM NaCl, 10mM Tris-HCl pH7.4, 5mM 2-mercaptoethanol. Final concentration of the particles was typically 400-500mg RNP/ml, equivalent to 200-250mg protein/ml (approximately 50000 cpm /µl).

Following concentration of RNP particles, they were microinjected into non-collagenased stage 2-4 oocytes (100nl/oocyte). The injected oocytes were then incubated for upto four days in modified Barths solution (53) to allow metabolism of the injected particles.

The piece of ovary containing the injected oocytes was washed in modified Barths solution, transferred to Tris buffered 3:1 medium (75mM KCl, 25mM NaCl, 10mM Tris-HCl pH7.2) and nuclei isolated manually from the injected oocytes under the dissecting microscope. Nucleoli were prepared in the same medium adjusted to 5mM $MgCl_2$ which aids polymerisation of actin, ensuring the integrity of the nucleoplasm thus decreasing protein loss (91).

Nuclei were transferred into centrifugation chambers made from bored glass discs sealed on the underside with round no.2 glass coverslips and containing 3:1 medium modified with $MgCl_2$, and the nuclear membrane removed from the nucleoplasm essentially as

described for the preparation of lampbrush chromosomes (179). Centrifugation chambers were covered with no.0 coverslips and centrifuged at 1200xg for 7 min in a sorvall RC-2B, HB-4 rotor in order to attach the preparations firmly to the no.2 coverslips. Following centrifugation, the no.0 coverslips were removed. The preparations were fixed in an ethanol series: 70% ethanol for 45 mins, 96% ethanol for 10 mins and 100% ethanol for 10min. The chambers were then immersed in xylene for 20 mins with gentle agitation to remove wax. The no.2 coverslips were then rinsed twice in acetone, mounted on slides, preparation upwards, with a small drop of Canada balsam and left on a hot plate to dry overnight.

The mounted preparations were dipped in fine grained nuclear emulsion (Ilford L4 nuclear research emulsion in gel form) diluted 1:1 with distilled water at 50°C and allowed to dry at 20° C with a constant draught for 1hr. The preparations were exposed to the photographic emulsion for 3-4 weeks at -70°C.

Following exposure, autoradiographs were developed for 10 min in Kodak D19 developer, washed in distilled water and fixed for 30 mins in Kodak Unifix. The slides were washed for 20 mins in tap water, stained with Giesma in phosphate buffer (50ml 10mM potassium phosphate buffer/ 4ml Giesma stain,BDH), flooded with tap water and rinsed in distilled water.

Photomicrographs were taken with a Wild MPS 51S camera fitted to a Leitz Ortholux microscope using Kodak Tri-X film.

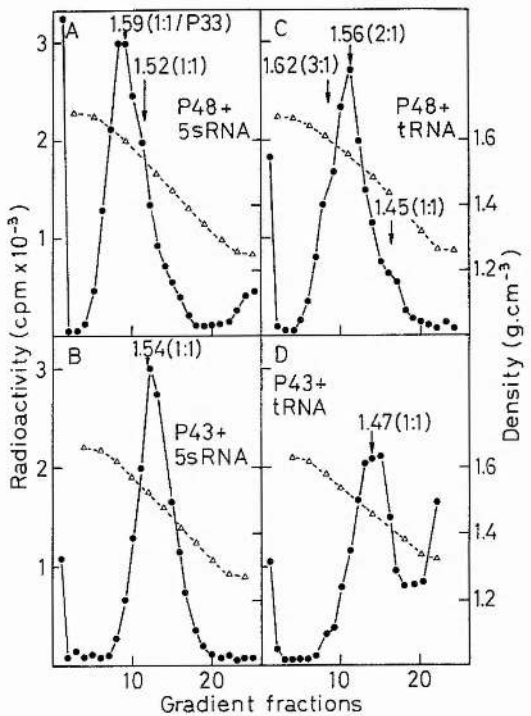
RESULTS

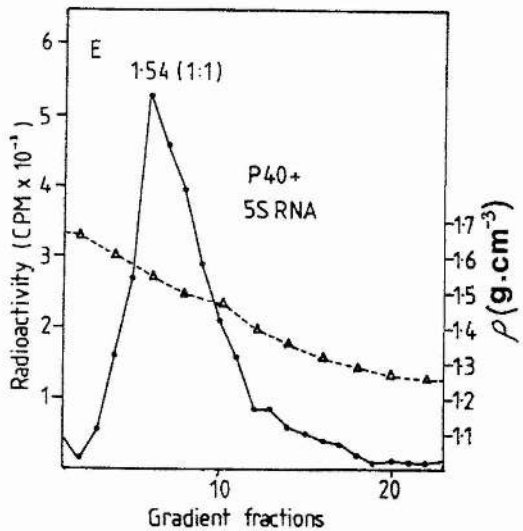
1:19 Specific Binding of RNA to Protein in vitro

The specificity of interaction of proteins with RNA molecules can be checked directly by isolating the different components and recombining them under conditions of controlled renaturation (66,87). After ^{125}I-labelled RNA is mixed with a 5-10 fold molecular excess of proteins, any ribonucleoprotein complexes formed can be analysed on CsCl density gradients after fixation of the complexes with formaldehyde. In all four combinations of the two RNAs with the two proteins, more than 70% of the radioactive RNA was incorporated into ribonucleoprotein complexes showing discrete density peaks (Fig.1). These features are characteristic of specific RNA-protein interaction, and do not relate to protein precipitation or simple charge interaction (66,87).

According to criteria established previously (66), the interaction of 5S RNA with P43 generates a ribonucleoprotein density peak (Fig.1B) near to 1.54g/cm^3, which is the theoretical value for a single RNA molecule of Mr 40000 with a single protein molecule of Mr 43000 (88). All ribonucleoprotein complexes described here which are formed by in vitro reconstitution have sedimentation values of 5-8S and thus are presumed to consist of only one protein molecule plus bound RNA rather than larger protein aggregates. The interaction of 5S RNA with P48 (Fig.1A) appears to produce complexes denser than expected from a 1:1 interaction (at 1.52g/cm^3). Only a shoulder of radioactivity occurs at the expected position in the gradient. Nevertheless, the observed peak is close to the value (1.59g/cm^3) expected from a 1:1 interaction of 5S RNA with the cleavage product

Fig.1. CsCl density gradient analysis of ribonucleoprotein complexes formed by renaturing isolated protein in the presence of purified RNA. The mass ratio of protein:RNA is 10:1 (20ug protein:2ug RNA). (A) mixture of P48 and 5S RNA. Arrows indicate theoretical postions of 1:1 molecular interaction with whole P48 and with the P33 cleavage product. (B) P43 with 5S RNA showing theoretical postions of 1:1 molecular interaction. (C) P48 with tRNA, showing theoretical postion of 3:1, 2:1 and 1:1 (RNA:protein) molecular interactions. (D) P43 with tRNA, showing theoretical postion of 1:1 molecular interaction. (E) P40 with 5S RNA, showing theoretical postion of 1:1 molecular interaction. (●) RNA radioactivity; (Δ) density of CsCl. Analysis of sedimentation of reconstituted material in sucrose gradients shows that the complexes of the type described here sediment at 5-10S. A negative control for non-specific interaction is provided by the mixture of P40 with tRNA which forms no stable complex in this assay (data not shown).


A
1.59(1:1/P33)
1.52(1:1)
P48+
5sRNA
C
1.56(2:1)
1.62(3:1)
P48+
tRNA
1.45(1:1)
B
1.54(1:1)
P43+
5sRNA
D
P43+
tRNA
1.47(1:1)
Radioactivity (cpm x 10⁻³)
Density (g.cm⁻³)
Gradient fractions


E
1·54 (1:1)
P40+
5S RNA
Radioactivity (CPM x 10⁻¹)
ρ(g.cm⁻³)
Gradient fractions

of P48, P33 (see Section 1:25). Indeed, the conditions of incubation used to form the ribonucleoprotein complexes do accelerate the cleavage of protein isolated as an electrophoretic band of Mr 48000. Furthermore, a naturally occurring 5S RNA/P33 particle does appear in later stages of oogenesis (see Section 1:24).

As reported previously, for Triturus 42S particle components (66), tRNA interacts with P45 (P48 of Xenopus) to produce a range of density particles which correspond to the binding of 1, 2 and 3 molecules of tRNA (Fig.1C) with a saturation level for the conditions described of 2-3 RNA molecules/ protein molecule. Using components from the Triturus 42S particle (66), no stable complexes were formed between P39 (corresponding to P43 of Xenopus) and tRNA. With components from Xenopus 42S particles, complexes were formed which correspond to a 1:1 interaction (Fig.1D) with a tendency to form nonspecific complexes as low density material at the top of the gradient. However, this aggregated material contained less than 20% of the RNA radioactivity.

From these results it is apparent that ribonucleoprotein complexes can be formed between either of the RNA species and either of the proteins.

Transcription factor TFIIIA (P40) also binds 5S RNA (62) and is confirmed in this analysis to form an RNP complex with a density of 1.54g/cm^3. In this analysis, 5S RNA taken from two different sources was used (from both 42S and 7S particles) with no difference in the bouyant density of the RNP complex or percentage of radioactive RNA bound. TFIIIA does not bind tRNA.

1:20 Effect of EDTA and High Salt on the Stability of 42S Particles.

Treatment of ribosomes with either low concentrations (2-10mM) of EDTA (92), or high concentrations (0.5M) of KCl (46) brings about dissociation into subunits and the release of a ribonucleoprotein complex containing 5S RNA and protein. A similar procedure can be adopted to analyse the internal organisation of 42S particles.

Treatment of 42S particles with 5mM EDTA results in the conversion of most of the material to slower sedimenting forms. Absorbance peaks at 14 and 7S are formed (Fig.2A). Analysis of protein and RNA constituents shows that, whereas the material sedimenting at 14S contains a mixture of all four major components, the 7S peak contains only P48 and 5S RNA at a molecular concentration at least five times greater than tRNA (Fig.2B). This result would suggest that in the 42S particle, at least some of the 5S RNA is complexed with P48 in a 1:1 ratio. The 42S particles used in this experiment were derived from stage 1 oocytes which contained little of the P33 breakdown product of P48.

Dialysis from buffer containing 30mM NaCl to buffer containing 0.5M NaCl also causes the breakdown of 42S particles, but here the breakdown is more complete, resulting in forms, all of which sediment at about 7S (Fig.3A). Since resolution of individual ribonucleoprotein complexes on sucrose gradients was unsatisfactory, a profile of RNA radioactivity in salt dissociated particles was obtain by centrifugation of formaldehyde-fixed products in CsCl gradients. The 42S particles were derived from stage 1 oocytes labelled in vivo for 18hr with ^{3}H-uridine. Very little of the RNA

Fig.2. Treatment of 42S particles with EDTA. $2A_{254}$ units of 42S ribonucleoprotein particles were isolated from stage 1 oocytes and treated with EDTA to a final concentration of 5mM. Treated particles were layered on to a 14ml. 15-40% sucrose gradient containing 30mM NaCl, 10mM Tris-HCl pH7.4, 5mM EDTA and 2mM 2-Mercaptoethanol and spun at 36000 rpm for 42hrs at 4°C. (A) sucrose gradient showing dissociation products at 14S(2) and 7S(3). Fraction (1) corresponds to undissociated material which is pelleted fraction (4) to the supernatant. (B) analysis of protein and RNA components from the fractions shown in (A). The Coomassie-stained protein gel shows presence of both P48 and P43 in the 14S material but only of P48 in the 7S material. RNA gels stained with acridine orange showed the presence of both 5S RNA and tRNA (in a 1:2 molecular ratio) in the 14S material but almost exclusively 5S RNA in the 7S material. (+) presence; (-) absence of RNA.

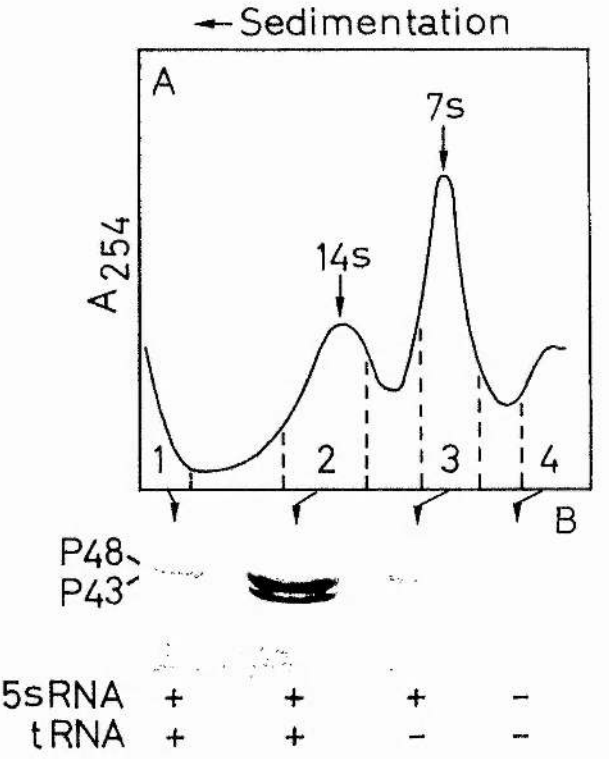
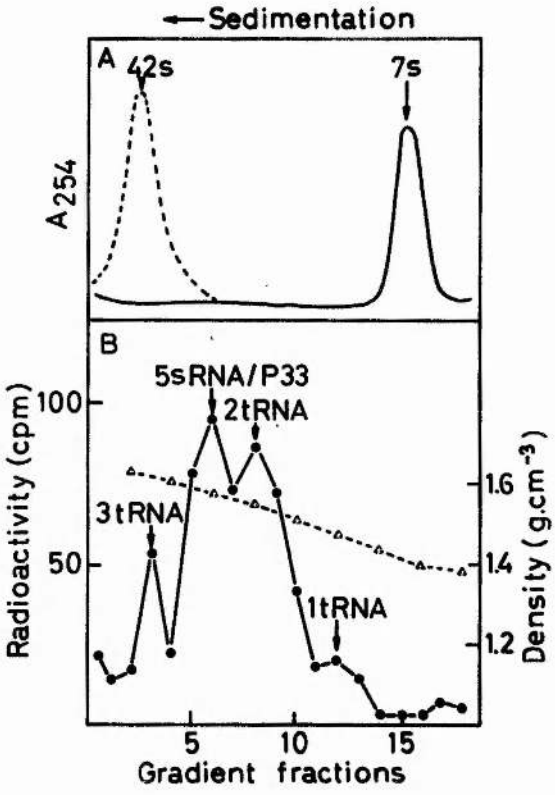

←Sedimentation
A
7s
14s
A254
1
2
3
4
B
P48
P43
5sRNA + + + −
tRNA + + − −

Fig.3. Treatment of 42S particles with 0.5M NaCl. $2A_{254}$ units of 42S ribonucleoprotein particles isolated from stage 1 oocytes were dialysed against buffer containing 0.5M NaCl for 16hr at 4° C. (A) sucrose gradient showing dissociation products as a single peak sedimenting about 7S. The position of the original 42S peak is shown (broken line). (B) CsCl density gradient analysis of ribonuceoprotein complexes contained in the 7S peak. Relative amounts of RNA are indicated by radioactivity (the ovary was originally labelled with ^{3}H-uridine as described in Section 1:4). The theoretical postion of complexes formed between 3,2 and 1 molecules of tRNA and a single protein and between 1 molecule of 5S RNA and P33 are indicated. (●) RNA radioactivity; (Δ) density of CsCl.


Sedimentation
A
42s
7s
A_{254}
B
5sRNA/P33
2tRNA
3tRNA
1tRNA
Radioactivity (cpm)
100
50
1.6
1.4
1.2
Density (g.cm^{-3})
5
10
15
Gradient fractions

label is recovered as free RNA at the bottom of the tube (1.65g/cm^3, Fig.3B): over 95% of the label is removed from the middle of the gradient in the form of stable ribonucleoprotein complexes. These complexes are detected as four peaks of radioactivity which correspond in density to those expected from single protein molecules in combination with 1, 2 and 3 molecules of tRNA, and with 1 molecule of 5S RNA. Due to the fixation procedure, the protein components of individual peaks cannot be identified. Nevertheless, the peak at the density position of 1.58g/cm^3 corresponds to the value expected from 5S RNA in combination with the P33 cleavage product of P48 (the content ratio of P33:P48 in the 42S preparation used for this particular experiment was about 1:1). Although the peak with lowest density (1.47g/cm^3) could contain either P48 or P43 in combination with a single tRNA molecule, the remaining two peaks at 1.55g/cm^3 and 1.62g/cm^3 are more likely to contain P48 in combination with 2 and 3 molecules of tRNA, respectively, since only this protein was found to be capable of multiple binding of tRNA in in vitro recombination studies (Fig.1).

1:21 Heterogeneity in Composition of 42S Particles

The static picture of 42S particles is one of a tetrameric complex of the basic monomeric unit comprising 2 molecules of P48, 1 molecule of P43, 1 molecule of 5S RNA and 3 molecules of tRNA. This picture does not incorporate any view of the particle being a dynamic particle possibly influencing transcription of 5S RNA or tRNA and the processes of rRNA transcription and ribosome assembly. However, some heterogeneity in the 42S particles is observed when fractions are

taken across the 42S region of sucrose gradients and in ovaries taken at different stages of development.

When the material that sediments in the 42S region of a sucrose gradient is separated into three fractions corresponding to the heavy side, middle and trailing side of the 42S peak (Fig.4), compositional variation is observed. Polyacrylamide gel electrophoresis shows that P43 is underrepresented in the fraction taken at the heavy side of the 42S peak. The gel which is stained with silver salts, also shows the RNA components which are recognized as negatively staining bands. It can be seen that the proportions of 5S RNA and tRNA components is similar in all three fractions. These observations would suggest that there is not a simple relationship within the particles of one type of RNA with one protein and the other type of RNA with the other protein.

Further heterogeneity is observed when 42S particles are isolated from ovaries at different stages of development. This heterogeneity can be seen with respect to both protein and RNA. Fig.5 shows the relative abundance of proteins in 42S particles isolated from ovary that contained: (1) only stage 1 (clear) oocytes, (2) mainly stage 2 (white) oocytes, and (3) mainly post-stage 2 (yolky) oocytes. Although the contribution of 42S material is mainly from stages 1-2 (65,93), it can be seen that protein composition changes from being mostly P48 in early ovary to being mostly P43 in more mature ovary. In addition, RNA from the three preparations was measured by scanning gels containing electrophoretically-separated RNA species. This analysis showed the ratio in A_{254} of 5S RNA to tRNA changed from 1.51 to 0.23 on going

Fig.4. Analysis of fractions across the 42S ribonucleoprotein peak isolated from stage 1 oocytes. (A) sucrose gradient profile showing fractions collected from the leading side(1), Middle(2) and trailing side(3) of the 42S peak. In material from this early oogenic stage, 42S material exceeds that of ribosomes(80S) and small ribonucleoprotein complexes(7S). (B) equal volumes of the 42S fractions were precipitated with ethanol and prepared for SDS-polyacrylamide gel electrophoresis. Staining with silver salts results in dark protein bands and negatively-stained RNA bands. (Pm) indicates proteins associated with mRNA which cosediment with 42S particles as mRNP particles(203). P33 is a cleavage product of P48 (see text).

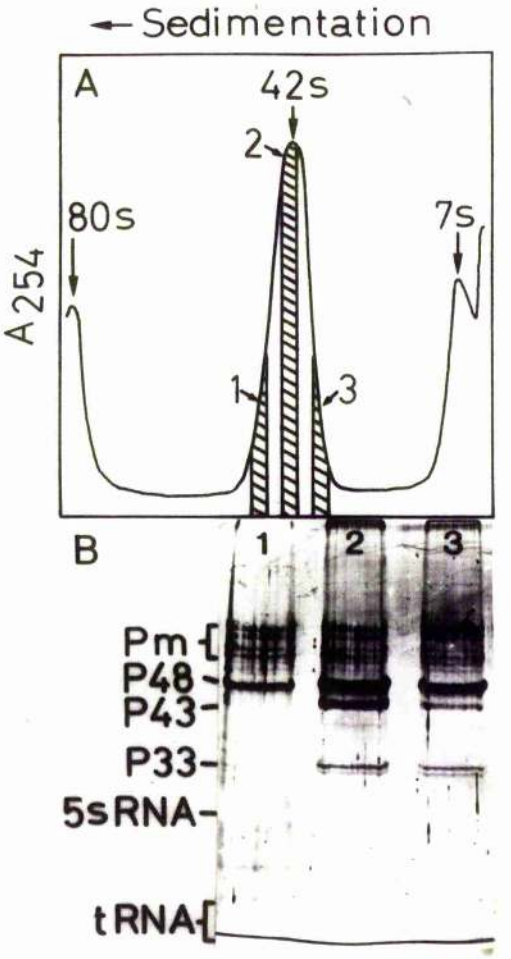
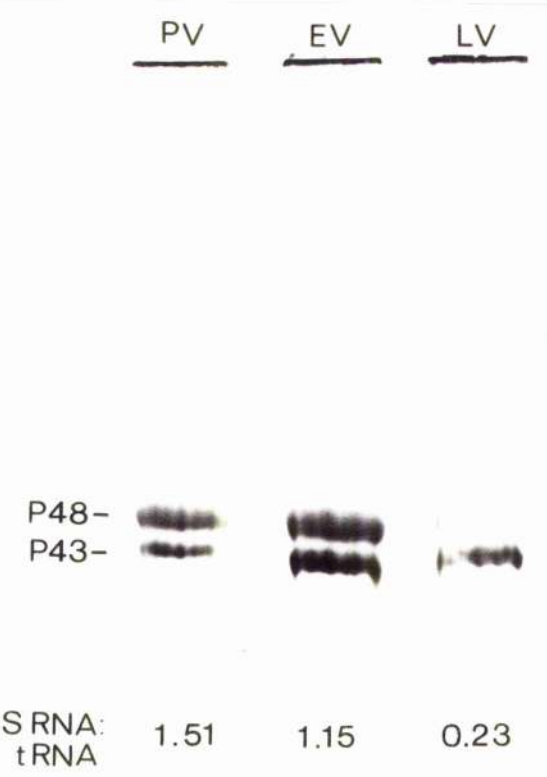

Sedimentation
A
42s
2
80s
7s
A 254
1
3
B
1
2
3
Pm
P48
P43
P33
5sRNA
tRNA

Fig.5. Analysis of 42S ribonucleoprotein particles at different stages of maturity. Animals were selected for ovaries having (1) exclusively stage 1 oocytes; (2) mainly stage 2 oocytes and (3) many post-stage 2 oocytes. The 42S RNP preparations were split for protein and RNA analysis. A Coomassie-stained protein gel is shown. RNA ratios were calculated from the area under 5S RNA and tRNA peaks in A_{254} scans of tube gels.


PV
EV
LV
P48-
P43-
5S RNA:
tRNA
1.51
1.15
0.23

from the samples from early stages to the samples from more mature stages. These values are equivalent to a range in molecular ratio of 1:1 to 1:6 (5S RNA:tRNA). Although the relative intensity in staining of P48 to P43 covers a similar (6 fold) range, this does not necessarily mean that there is a one to one molecular ratio relationship between 5S RNA and P48 and between tRNA and P43. For instance, in 42S particle preparations from ovary containing only early stage 1 oocytes (up to 50μm diam.), the protein composition is often almost exclusively P48, yet both 5S and tRNA are well represented (see also Fig.4.B, track 1).

The above results demonstrate that heterogeneity in the particle composition exists within a single 42S RNP preparation, and heterogeneity also arises in 42S RNP particles taken from ovaries from different developmental stages. These changes reflect the dynamic state of the 42S particles.

1:22 Amino Acid and Peptide Analysis of P43, P48 and P40

Ribonucleoprotein particles sedimenting at 42S and 7S were isolated on sucrose gradients. Ethanol precipitation followed by SDS polyacrylamide gel electrophoresis reveals that the major protein components of 42S particles are proteins of molecular weights 48KDa and 43KDa, whilst the major protein component of the 7S particle is a protein of 40KDa. In our experience, these three proteins were poorly soluble in the absence of RNA, SDS below 0.1% and/or 8M urea.

The relationship between these proteins needs to be established, in order that their possible effect on the transcription of 5S and tRNA genes, and the formation, distibution and utiliization of 42S particle components may be determined.

For this analysis, proteins were isolated from an SDS polyacrylamide gel, by excising the region containing the individual proteins and then eluting the proteins in elution buffer containing SDS. Purity of the eluted proteins was checked by electrophoresis.

2D electrophoresis of these proteins show them to have a basic charge, with P40 and P43 being difficult to resolve (93). Similar proteins are contained in 7S and 42S particles of the fish, Tinca tinca and it was suggested that the 7S particle protein was a cleavage product of the lower molecular weight protein of the 42S particles (57). The aim of these experiments was to determine if there was any homology between the three proteins.

Amino acid analysis was carried out on three different preparations of the proteins. The results are presented in Table 2. From this data no protein of the three could be produced by proteolytic cleavage from any other of the three proteins. This is because P40 contains more tyrosine than P43 or P48 as well as other differences between the other two proteins and P43 contains more leucine, lysine, histidine and arginine than P48.

Table 2. Amino acid analysis of P48, P43 and P40. Approximately 50ug of protein isolated from preparative SDS-polyacrylamide gels was dialysed against distilled water and hydrolyzed in 6N HCl, 0.1M thioglycolic acid at 110°C for 24hr under an atmosphere of N_2. The residues were analysed using an automated single column analyzer.

NO. OF RESIDUES PER MOLECULE

	P48	P43	P40
Asp	40	22	39
Thr	26	21	19
Ser	37	30	26
Glu	44	32	40
Pro	19	18	13
Gly	57	39	43
Ala	43	29	28
Val	36	23	22
Cys(½)	6	10	3
Met	6	4	3
Iso	24	8	9
Leu	33	34	25
Tyr	6	7	13
Phe	19	17	15
Lys	24	29	24
His	10	21	16
Arg	18	29	28
Trp	ND	ND	ND
% Basics (Arg, Lys and His)	12.2	20.7	19.2

The lack of relatedness is further demonstrated by the cyanogen bromide cleavage pattern of the three proteins. Cyanogen bromide cleavage was carried out on the gel purified proteins. This compound cleaves proteins at the peptide linkage between methionine and its neighbouring amino acid. The results are shown in Fig.6. (This gel is stained with silver and the peptides do not necessarily appear in stochiometric amounts.) There is no similarity between these three cleavage patterns of P48, P43 and P40.

1:23 Immunological analysis

Immunological analysis using the technique of Western blotting (89) is also a sensitive assay to test for relatedness. The proteins in question are run out on an SDS gel, transferred to nitrocellulose and reacted with serum against one of the proteins. The bound antibody is detected by the binding of ^{125}I-labelled protein-A which binds to most classes of IgG from different animals including rabbit. Our antibodies were raised in rabbits against the gel purified proteins as an RNP complex with yeast tRNA. From Fig.7 it can be seen that anti-P48 reacts strongly with P48 taken from 42S particles, but with no protein from the 7S region of a sucrose gradient. Likewise anti-P43 only reacts with P43 from 42S particles, whereas anti-P40 only reacts with P40 from the 7S region. These antibodies are highly specific for their respective proteins. This indicates that these three proteins are dissimilar antigenically.

Fig.6. Cyanogen bromide cleavage patterns of P48, P43 and P40. 5-10ug of protein was reacted with 100 molar excess of cyanogen bromide for 24-48hr at room temperature. Staining intensity with silver salts is not necessarily proportional to the amounts of material in the bands. M, marker polypeptides with Mr values (times 10^{-3}).

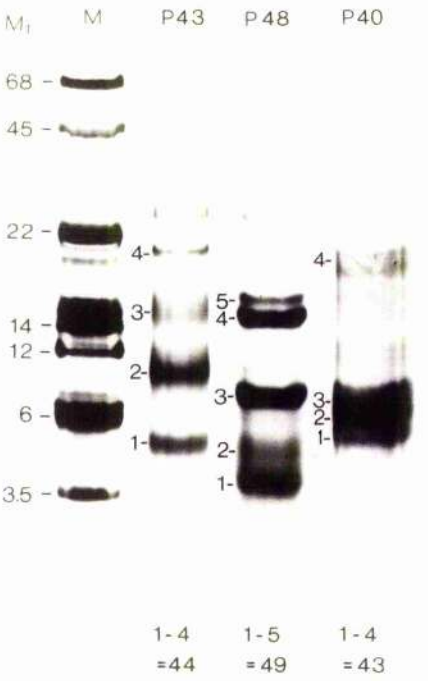
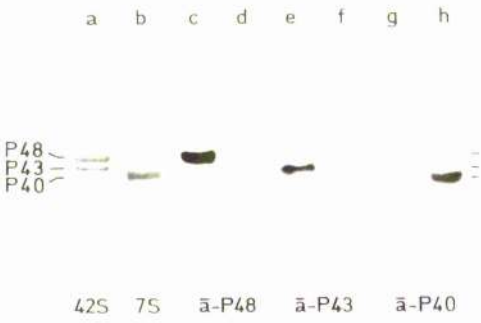
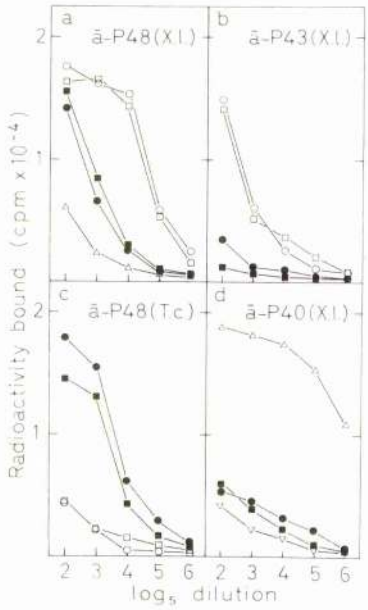

M_1
M
P43
P48
P40
68
45
22
14
12
6
3.5
4-
3-
2-
1-
5-
4-
3-
2-
1-
4-
3-
2-
1-
1-4
=44
1-5
=49
1-4
=43

Fig.7. Radioimmunostaining of 42S and 7S RNA particle polypeptides using antibodies directed against P48, P43 and P40 with labelled protein-A. (a) 42S particle proteins stained with Coomassie Blue; (b) stained 7S particle proteins; c,d transfers of proteins shown in a,b, immunostained with anti-P48; e,f, transfers immunostained with anti-P43; g,h, transfers immunostained with anti-P40.

Fig.8. Radio-immunoassays showing the capacties of RNP particles from Xenopus and Triturus species to bind antibodies directed against P48, P43 and P40. Wells of a microtitre plate were coated with antigen at 10µg/ml. Antisera used at 1:20 to (a) P48 from X.laevis; (b) P43 from X.laevis; (c) P48 from T.cristatus; (d) P40 from X.laevis were assayed for binding to 42S particles from O, X.laevis; □, X.borealis; ●, T.cristatus and ■ T.vulgaris; and to △ X.laevis; ▽ X. borealis 7S RNP particles.


a b c d e f g h
P48
P43
P40
42S 7S ā-P48 ā-P43 ā-P40


a
ā-P48(X.l.)
b
ā-P43(X.l.)
c
ā-P48(T.c.)
d
ā-P40(X.l.)
Radioactivity bound (c.p.m x 10⁻⁴)
log₅ dilution

42S and 7S particles have been isolated from a range of amphibians and teleost species (58). Cross reactivity of these proteins were tested by radioimmunoassay using native RNP particles, and by Western blotting from SDS gels. Using the antibodies raised against the X.laevis proteins, cross reactivity was tested against proteins from the 42S and 7S region of X.borealis, Triturus cristatus and Triturus vulgaris. The technique used is radioimmunoassay (RIA) and, while similar to Western blotting in retarding the amount of ^{125}I-labelled protein-A bound to antibody-antigen complexes, differs in as much as the antigens used are non-denatured RNP particles attached to the wells of a 'sticky' plate.

As the curves in Fig.8 show, anti-P48 from X.laevis shows similar affinity and avidity of binding to 42S particles from X.borealis, whereas binding to the Triturus 42S particle is much reduced. A similar result is seen for anti-P43 binding with little or no binding to the Triturus 42S RNP for this antibody. For anti-P40, binding to any proteins of the 7S region from any species other than X.laevis is very much reduced. Also tested here was the antibody against T.cristatus, P48. This shows that like anti-P48 of X.laevis, antibodies directed against the Triturus protein react only within the same genus.

These results may be interpreted as a divergence between Xenopus and Triturus genera with respect to 42S particle proteins, and a divergence between X.laevis and its sub-species X.Borealis with respect to P40.

Consistent with these interpretations are the results of radioimmunostainings by Western blotting (Fig.9). The protein from the 42S and 7S regions of a sucrose gradient were precipitated, and run out on SDS gel, then transferred to nitrocellulose, where they may become partially renatured. However, the antibodies have similar affinity of binding as shown by RIA, and it is clear that the X.laevis anti-P48 recognises the homologous protein from X.borealis but not from T.cristatus or T.vulgaris. This is similar for anti-P43 (the X.borealis protein is apparently of slightly higher molecular weight). Similar results are obtained with the T.cristatus anti-P48, which only recognises its own antigen and that of T.vulgaris.

With anti-P40, however, only P40 from X.laevis is recognised. This is consistent with the RIA results. It is also true of X.borealis anti-P40 which does not crossreact with X.laevis P40 (P.J.Ford, pers. comm.). Thus the proteins that form a 7S RNP in the Xenopus species have diverged so much that they are no longer recognised by polyclonal antibodies against the equivalent protein from the other Xenopus species.

The existence of a 7S RNP in the Triturus species is doubtful, since no RNP peak is observed on sucrose gradients from previtellogenic ovary; there is no major protein in the 7S region of this gradient (Fig.10) and no 5S RNA is observed after phenol extraction (data not shown). This does not exclude the possibility of a protein similar to P40 of Xenopus being present in much smaller amounts, but certainly there is no storage function if present. The situation in Triturus seems to be much simpler than Xenopus. Why should Xenopus have two storage particles? These findings obviously

Fig.9. Radio-immunostaining of RNP particle proteins from Xenopus and Triturus species using antibodies directed against P48, P43 and P40. (a) 42S particle proteins from X.laevis, X.borealis, T.cristatus, and T.vulgaris immunostained using antiserum to P48 from X.laevis. (b) 42S particle proteins as in (a) immunostained using antiserum to P43 from X.laevis. (c) 42S particle proteins as in (a), immunostained using antiserum to P48 from T.cristatus; (d) 7S particle proteins from X.laevis and X.borealis and 42S particle proteins from T.cristatus and T.vulgaris immunostained using antiserum to P40 from X.laevis. Mr, molecular weight $x10^{-3}$ of marker polypeptides.

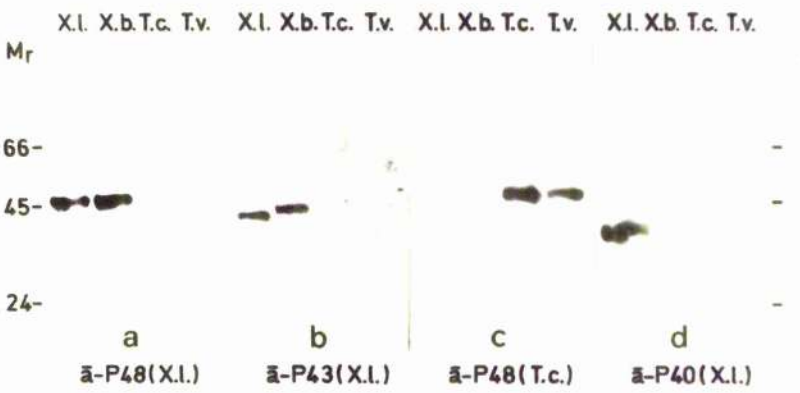
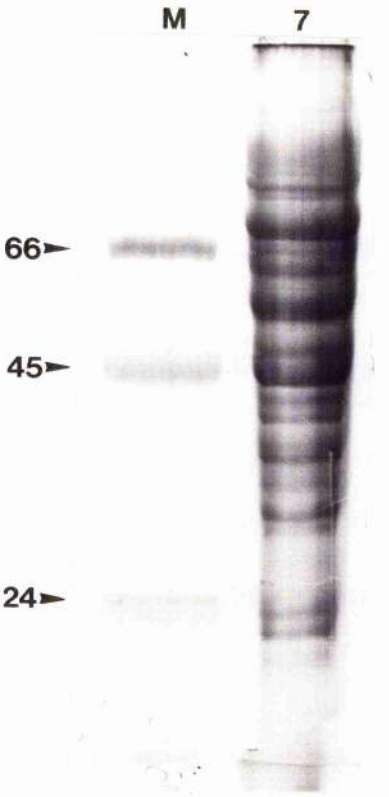

X.l. X.b. T.c. T.v.
X.l. X.b. T.c. T.v.
X.l. X.b. T.c. T.v.
X.l. X.b. T.c. T.v.
Mr
66-
45-
24-
a
ā-P48(X.l.)
b
ā-P43(X.l.)
c
ā-P48(T.c.)
d
ā-P40(X.l.)

Fig.10. A Coomassie stained polyacrylamide gel showing the protein profile of the proteins sedimenting at 7-10S from T.cristatus oocyte homogenate (7). No single major staining band at equivalent Mr for the Xenopus transcription factorIIIA is observed. Mr, molecular weight $x10^{-3}$ of marker polypeptides.


M
7
66
45
24

mean that caution should be applied to generalising about events involving these proteins, including that of in vitro transcription assays, where many heterologous components are used (94,95).

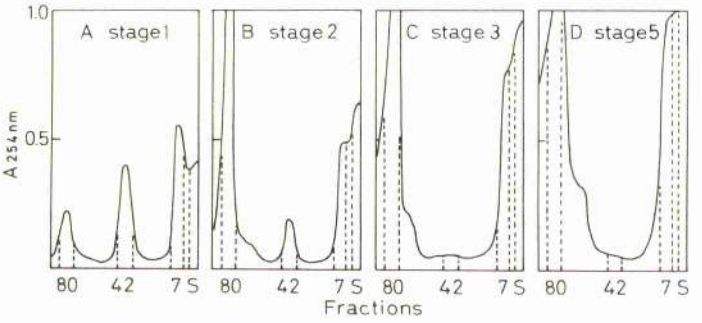
1:24 Patterns of Protein Presence in Storage Particles during Oogenesis

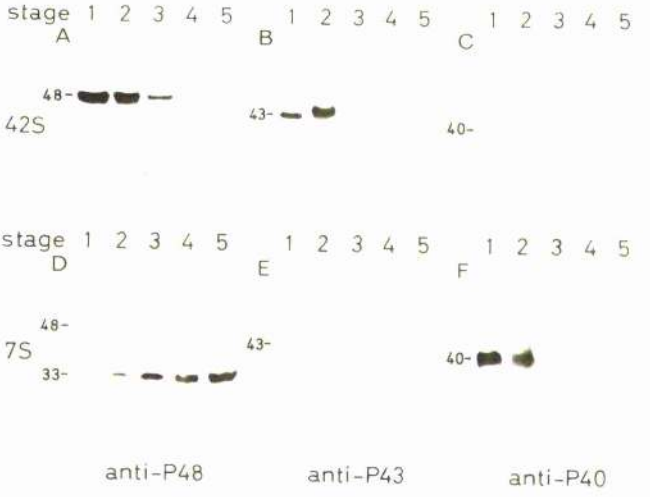
The results in Section 1:20 demonstrate 42S particle heterogeneity in ovaries containing a range of different developmental stages of oocytes. Since the 42S particles are contributed mainly by oocytes in stages 1 and 2 (65,93), it is important to determine the content of the three RNA-binding proteins in oocytes of each particular developmental stage within the same ovary.

Ovary was dissected from maturing Xenopus laevis, and oocytes were released from the tissue by treatment with collagenase as described in Section 1:4. Individual oocytes were grouped according to size and colour, and non-overlapping categories were selected as typical of the developmental stage described by Dumont (77): stage 1, clear (0.1-0.2mm); stage 2, white (0.3-0.4mm); stage 3, fawn (0.5-0.6mm); stage 4, black/green (0.7-0.8mm); stage 5, brown/green (1.0-1.2mm). Groups of 400 oocytes from each stage were homogenized and fractionated by centrifugation into 100xg pellets, 3000xg pellets, 10000xg pellets and 10000xg supernatants, as described in Section 1:4. The 10000xg supernatants were layered on sucrose gradients and fractionated further into components sedimenting at 80S, 42S, 7S and less than 7S (supernatants). The fractions collected and used are indicated in Fig.11. Proteins were extracted

Fig.11. Preparative sucrose gradient separation of homogenates of oocytes taken at different developmental stages. Low speed pellets were taken from homogenates as described in Section 1:4 and the supernatants were further fractionated into: ribosomes(80); 42S RNP particles(42); 7S RNP particles(7) and gradient supernatant(S) as shown. The traces of absorbance represent relative amounts of material from 400 oocytes at stage 1 (A), Stage 2 (B), stage 3 (C) and stage 5 (D). The trace for stage 4 oocytes is essentialy intermediate between (C) and (D).

Fig.12. Immunoblotting of from 42S particles and 7S particles with antibodies directed against the particle proteins P48, P43 and P40. Fractions from homogenates of oocytes at different stages taken as indicated in Fig.11. Each fraction was divided into four (equivalent to 100 oocytes) and proteins were electrophoresed and electrotransferred to nitrocellulose as described in Section 1:9. After binding of antibodies the transfers were reacted with ^{125}I-labelled protein-A. The autoradiographic images are shown for: 42S particle proteins from stage 1 to 5 reacted with anti-P48 (A), anti-P43 (B) and anti-P40 (C); and 7S particle proteins from stage 1 to 5 reacted with anti-P48 (D), anti-P43 (E) and anti-P40 (F). Postions of the major ataining proteins P48, P43, P40 and the cleavage product P33 (see text) are indicated.


1.0
0.5
A 254 nm
A stage1
B stage2
C stage3
D stage5
80 42 7S
Fractions


stage 1 2 3 4 5
A
B
C
48-
43-
40-
42S
D
E
F
7S
33-
anti-P48
anti-P43
anti-P40

from each fraction, separated, in triplicate, by SDS/polyacrylamide gel electrophoresis, transferred to nitrocellulose and immunoblotted using the antibodies raised against each of the 5S RNA-associated proteins, P48, P43 and P40. Antibody binding was assayed by reaction of the immunoblots with ^{125}I-labelled protein-A followed by autoradiography. Components were identified by comparison with sample tracks stained with Coomassie blue. Unless otherwise stated, each track represents the reaction given by material from 100 oocytes. The limit of detection by the procedures used is approximately 10ng protein.

Fig.12 shows autoradiographs representing levels of binding of ^{125}I-labelled protein-A to immunoblots of 42S RNP protein and 7S RNP proteins reacted with anti-P48, anti-P43 and anti-P40. In this analysis, P40 is detected only in material sedimenting at about 7S, and mainly from oocyte stages 1 and 2. This is similar to results reported elsewhere (96), where the level of this protein increased only slightly during stage 1 through to stage 2, and declined to less than 5% of its peak value by stage 6. However, this other analysis was done on total oocyte homogenates and not on RNP particles separated into sedimentation classes as here.

Of the 42S RNP particle proteins, P43 is detected only in the 42S fraction isolated from stages 1 and 2. The other 42S RNP protein, P48, is detected also during stages 1 and 2, and generally also into stage 3. These results are consistent with the pattern of appearance and disappearance of the 42S and 7S RNP storage particles (65). In addition to detection of P48 in 42S particles, anti-P48 reacts with a protein or Mr 33000 (P33) in the 7S fraction. In

contrast to the P48 pattern, P33 appears at stage 2 and increases in amount up to the end of the oogenic period. In general there is a reciprocal relationship between the relative amounts of P48 in 42S particles, and of P33 in 7S particles.

None of the proteins contained in gradient fractions sedimenting at less than 7S (the supernatants in Fig.11) reacts in the immunoblot assay with any of the three antibodies (data not shown). This is true of all oogenic stages and also of supernatants from homogenates of total ovary containing very small (<0.1mm) oocytes. It must be concluded that at no time during oogenesis are there substantial amounts of soluble P48, P43 or P40.

The above results relate to morphologically distinct stages. However, it was of interest to determine whether these proteins could be detected very early in oogenesis where the stage 1 oocytes are less than 50μm in diameter, as this may have a bearing on ascribing functions to these proteins in relation to the early regulation of 5S and tRNA synthesis. For this analysis, ovaries from twenty Xenopus at 3 months postmetamorphosis were processed as described above, to isolate 42S and 7S RNP particles (Fig.13). These fractions were divided into three and analysed using the immunoblotting technique with anti-P48, anti-P43 and anti-P40. The results are shown in Fig.14.

Using anti-P43, no protein bands can be seen reacting with this antibody, indicating that no P43 is present at this stage, or at least is below the level of detection using this method. P48, however, is detectable in the 42S region of this gradient, and also

Fig.13. Fractionation of RNP particles from immature oocytes. Homogenates of ovaries containing oocytes no greater than 50μm in diameter were separated on 15-40% sucrose gradients. A parallel sucrose gradient containing a homogenate of stage 1 oocytes was used to locate the postion of ribosomes(80), 42S particles(42) and 7S particles(7).

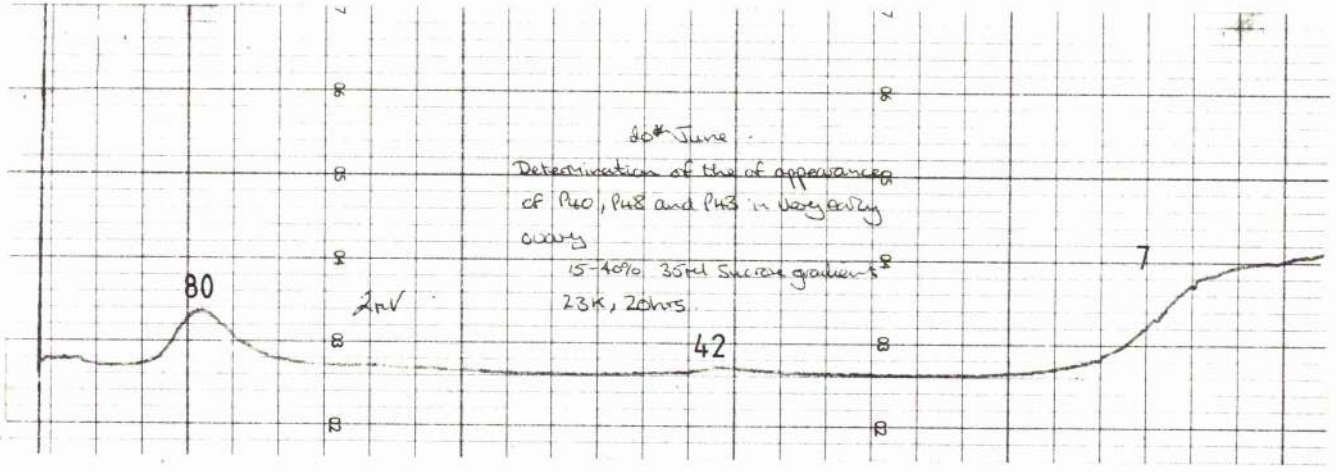
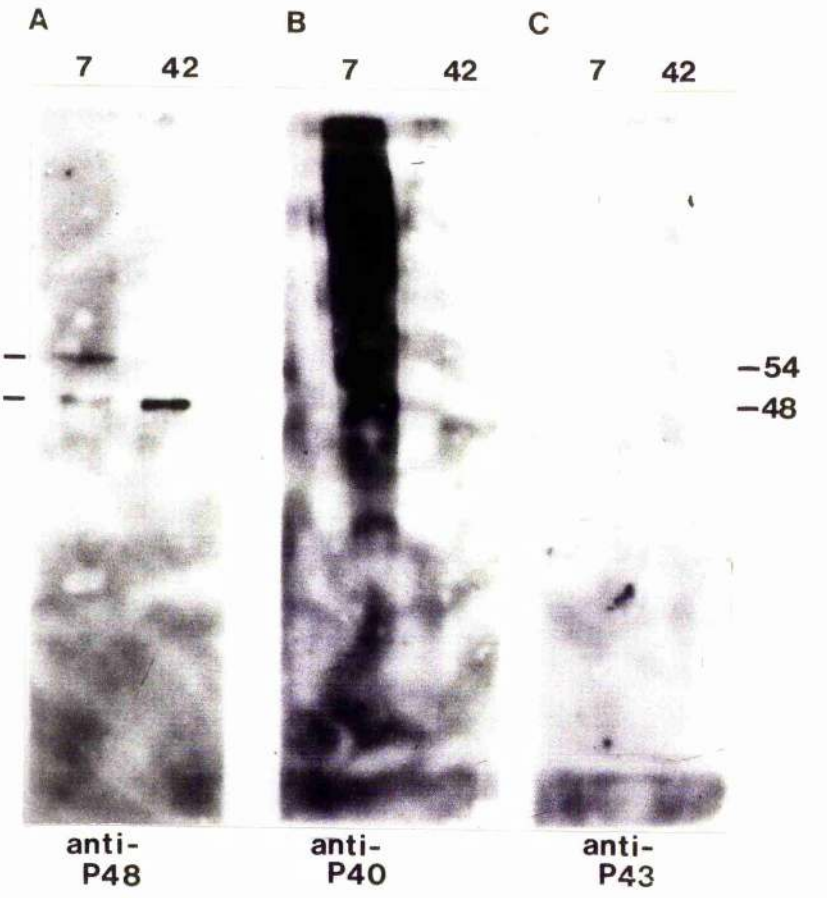

80
2nV
20th June
Determination of the of appearances
of P40, P48 and P43 in very early
ouary
15-40% 35ml Sucrose gradient
23K, 20hrs.
42
7

Fig.14. Immunoblotting of proteins from 42S particles and 7S particles with antibodies directed against the particle proteins P48, P43 and P40. Fractions from a homogenate of oocytes less than 50um in diameter were taken as indicated in Fig.13. Proteins were electrophoresed and electrotransferred onto nitrocellulose as described in Section 1:9. After binding of antiboby the transfers were reacted with ^{125}I-labelled protein-A. (A) 42S particle and 7S particles immunoblotted with anti-P48; (B) 42S particles and 7S particles immunoblotted with anti-P43; (C) 42S particles and 7S particles immunoblotted with anti-P40. Molecular weight of immunostaining bands ($x10^{-3}$) are indicated.


A
7
42
B
7
42
C
7
42
54
48
anti-
P48
anti-
P40
anti-
P43

in the 7S region. P40 is not detectable in the 42S region, or in the 7S region. It would appear that, whatever their functions, P48 becomes more abundant sooner than P40 or P43 and the composition of 42S and 7S particles at very early oogenesis is different from that found at mid stage 1 and at stage 2.

1:25 Proteolytic Cleavage of P48

It has been reported previously (65) that excess 5S RNA persists in late oocytes in the form of an RNP particle. Here it is demonstrated that P33 apparently is accumulated in material sedimenting at about 7S. That P33 is associated with 5S RNA is suggested from two types of experiment. First, analysis of fractions across the 7S peak (data not shown) indicates a similar distribution of P33 and 5S RNA, and a different distribution of both from tRNA. Second, incubation of material from the 7S fraction of stage 5 oocytes with anti-P48 bound to protein A-sepharose beads results in the selective precipitation of 5S RNA (Fig.15). Incubation of the anti-P48 beads with the 7S fraction of stage 1 oocytes results in no precipitation of 5S RNA, presumably because the 5S RNA at this early stage is associated with P40 rather than P33.

When P48 (from Triturus) is isolated by gel electrophoresis and eluted from the gel in 0.1M NaCl, 1% SDS, cleavage of P48 to P33 (a variable amount) can be observed (Fig.16, lane 3). This fragmentation accounts for the observed protein/RNA binding data (see Fig.2A). Cleavage of P48 has also been observed in the isolated 42S particle (Fig.4).

Fig.15. Selective immunoprecipition of 5S RNA from stage 5 oocytes with anti-P48. RNA was extracted from sucrose gradient fractions containing approximately: 52ug 42S RNP particles from stage 1 oocytes (lane 3); 100ug of RNP material sedimenting about 7S from stage 5 oocytes (lane 5) and separated by gel electrophoresis. Equivalent amounts of material from: 42S particles (lane 2); 7S particles from stage 1 oocytes (lane 4); 7S material from stage 5 oocytes (lane 6) were incubated with anti-P48 linked to protein-A sepharose. The immunoprecipated material was used for RNA extraction and analysis of RNA species as for total RNA. After electrophoresis the gel was stained with ethidium bromide (10µg/ml) and photographed on an ultra-violet light box. The postions of 5S RNA and tRNA are indicated.

24 µg 7S RNP particles from stage 1 oocytes.

Fig.15. Selective immunoprecipition of 5S RNA from stage 5 oocytes with anti-P48. RNA was extracted from sucrose gradient fractions containing approximately: 52ug 42S RNP particles from stage 1 oocytes (lane 3); 100ug of RNP material sedimenting about 7S from stage 5 oocytes (lane 5) and seperated by gel electrophoresis. Equivalent amounts of material from: 42S particles (lane 2); 7S particles from stage 1 oocytes (lane 4); 7S material from stage 5 oocytes (lane 6) were incubated with anti-P48 linked to protein-A sepharose. The immunoprecipated material was used for RNA extraction and analysis of RNA species as for total RNA. After electrophoresis the gel was staired with ethidium bromide (10ug/ml) and photographed on an ultra-violet light box. The postions of 5S RNA and tRNA are indicated.

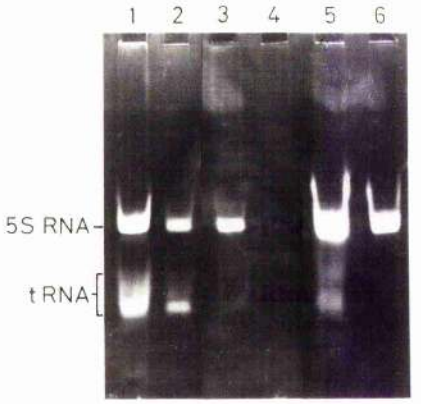
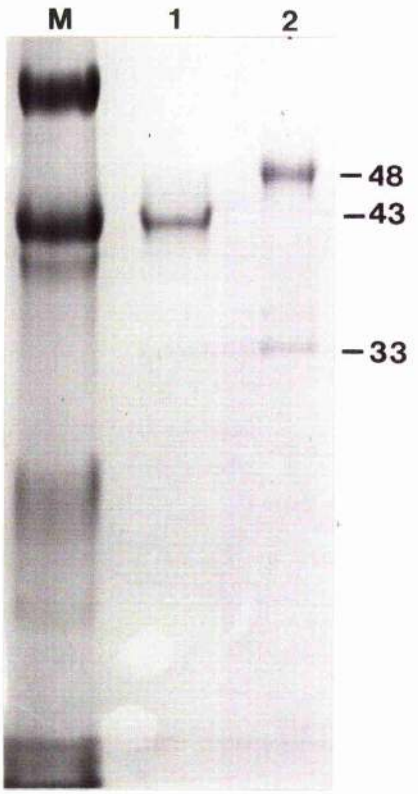

1
2
3
4
5
6
5S RNA
t RNA

Fig.16. Cleavage of P48 to P33 from isolated P48 from T.cristatus. 42S particles were prepared from oocytes of T.cristatus as described in Section 1:4. After separation of the 42S particle proteins by preparative gel electrophoresis, the proteins were located by staining a strip of the gel lane with Coomassie- blue. The bands were then removed from the preparative lanes and eluted in 0.1M NaCl, 1% SDS, 2mM MgCl and 5mM 2-mercaptoethanol for 16-24hr at room temperature. The eluted proteins were reprecipitated and run on an analytical polyacrylamide gel. lane 1, isolated P43; lane 2, isolated P48. Both proteins migrate slower after isolation due to salt present following reprecipition of the proteins from elution buffer. Molecular weight of bands (times 10^{-3}) indicated.


M
1
2
48
43
33

P33 can be derived from isolated 42S RNP particles by incubation of the particles with a supernatant fraction from homogenized stage 3 oocytes (Fig.17), and since this cleavage can be prevented by the addition of the protease inhibitor, PMSF (A.Lomas, personal communication), it seems reasonable to assume that P33 is produced from P48 through a specific proteolytic cleavage as a natural event of oogenesis. Occasionally, a second putative cleavage product of Mr 11000 is detected in 42S particles by immunoblotting using anti-P48.

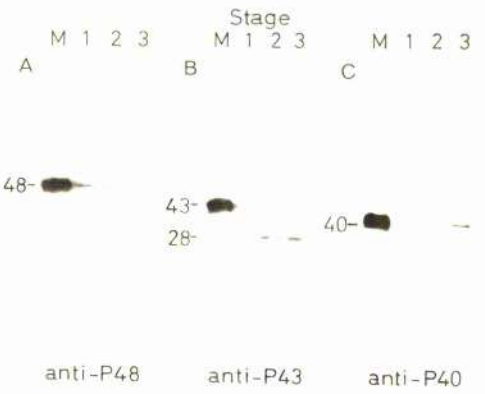
1:26 Presence of Storage Particle Proteins in the Nucleus

One cellular compartment in which 5S RNA associated proteins play a critical role in the metabolism of 5S RNA and probably tRNA is within the nucleus. P40 has already been characterised as a transcription factor (TFIIIA) of 5S RNA genes which has the dual ability to bind to the internal promotor and to the transcript of the same genes (94,97,98). In order to assess the significance of nuclear location, oocyte nuclei were isolated manually into a medium which causes gelling of the nucleoplasm (to prevent leaching of soluble components), and the nuclear proteins were immunoblotted using anti-P48, anti-P43 and anti-P40. Surprisingly, P40 was not detected as a major nuclear protein under conditions where P48 is detected as a nuclear protein, primarily during stages 1 and 2 (Fig.18A,C). During these stages the synthesis of 5S RNA and tRNA is a major feature of oocyte activity. It is reasonable to suppose that P48 plays a part in the nuclear metabolism of 5S RNA and possibly tRNA, either by 1) functioning directly, or as an auxiliary protein, in transcription of 5S RNA and tRNA genes; 2) stabilization of RNA

Fig.17. Production of an Mr-33000 polypeptide from isolated 42S RNP particles. Previtellogenic ovary was incubated with ^{14}C- labelled amino acids (10μCi/ml 57Ci/mol) as described in Section 1:4 and 42S particles were isolated as shown in Fig.11. Equal amounts (8μg) RNP as gradients fractions were incubated at 18°C for 18hr in the absence (lane 1) and presence (lane 2) of homogenate derived from five unlabelled stage 3 oocytes. Precipitated protein was analysed by fluorography of an SDS- polyacrylamide gel. Mr values (times 10^{-3}) for the labelled bands are indicated.


1
2
48
43
33

Fig.18. Immunoblotting of proteins from isolated nuclei with anti-P48, anti-P43 and anti-P40. (A) Reaction of anti-P48 with proteins from 60 nuclei of stage 1, 2 and 3 oocytes. lane M is the reaction with 42S particles used as marker. (B) as for (A) but using anti-P43. (C) as for (A) but using anti-P40 and 7S RNP as marker. Mr values (times 10^{-3}) for the labelled bands are indicated.


Stage
M 1 2 3
M 1 2 3
M 1 2 3
A
B
C
48-
43-
28-
40-
anti-P48
anti-P43
anti-P40

molecules within the nucleus; or 3) transport of RNA molecules to the cytoplasm.

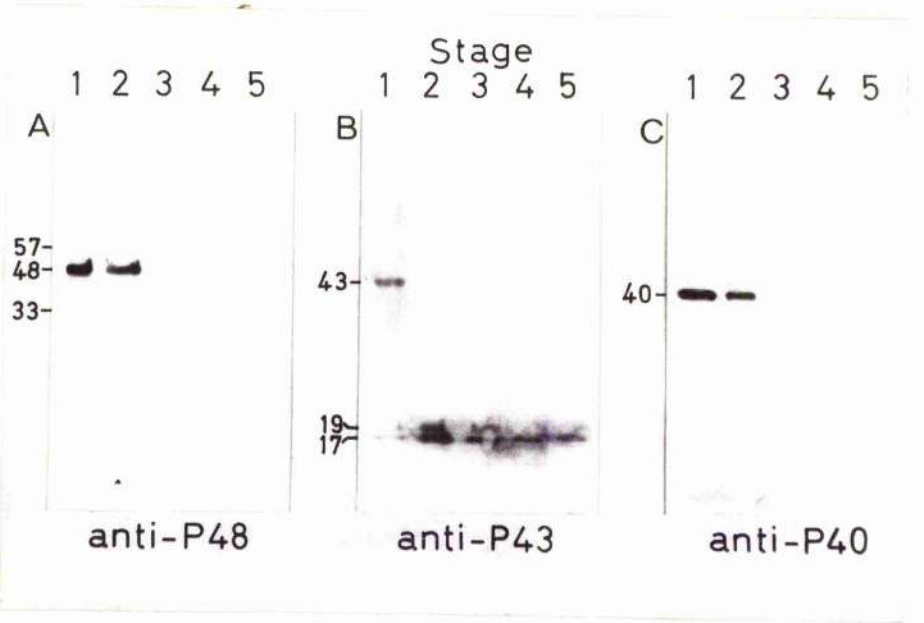
However, in this same analysis, P40 is detected later at stage 3 (Fig.18C) and in mature oocytes (99). P43 is not detected as a major nuclear antigen, yet anti-P43 binds a protein of Mr 28000 from the nuclear fraction of oocytes which have developed through stage 2 (Fig.18B). This observation will be discussed below.

1:27 Identification of Additional Proteins Which May Bind 5S RNA and tRNA

The ability of proteins to bind 5S RNA or tRNA may not be confined to the storage particle proteins described here. For instance, with respect to the ribosome of rat liver, several proteins have been identified with the ability to bind 5S RNA (100-102). The identification of such proteins is important because they may have an effect on transcription in addition to their role in the translation machinary.

Two experiments were performed to identify other proteins. First, total homogenates of oocytes were analysed by immunoblotting to check that the entire series of precursors and products of P48, P43 and P40 had been identified. The presence of proteins that bind anti-P40 is the simplest to describe, for in total homogenates the only protein detected is P40 itself, which is a major component only in stages 1 and 2 (Fig.19C), which is not entirely consistent with similar experiments carried out by other workers (103). Anti-P48 is bound by P48 in stages 1 and 2 and, occasionally, into stage 3. In

Fig.19. Immunoblotting of proteins from whole homogenates of oocytes with anti-P48, anti-P43 and anti-P40. 100 oocytes of each stage 1 to 5 were selected and homogenised. Glycerol to 10%, 2-mercaptoethanol to 5mM and SDS to 1% were added and the samples prepared for electrophoresis. (A) Reaction of anti-P48 with total oocyte protein, stages 1 to 5. (B,C) as (A) but using anti-P43 and anti-P40 respectively. Mr values (times 10^{-3}) for the labelled bands are indicated.


Stage
1 2 3 4 5
A
57-
48-
33-
anti-P48
1 2 3 4 5
B
43-
19-
17-
anti-P43
1 2 3 4 5
C
40-
anti-P40

addition, a cross-reacting protein of Mr 57000 appears at stages 2 and 3 (Fig.19A) but is lost later. (This later protein is presumably the one identified in pelleted fractions (Fig.19A).) The presumptive cleavage product of P48, P33 is poorly represented in oocytes taken from the animal used for this set of experiments (see 'Discussion'). In using anti-P43 with total homogenate protein, the only major species identified are P43 and a doublet at Mr 17000 and 20000. The lower band is maintained in intensity from stages 2 to 5 and has the characteristics of the putative cleavage product (P17) that is associated with ribosomes (see below). In all homogenates prepared from stages4 to 6 there may be some loss of material due to the necessity of removing the large amounts of yolk by sedimentation (50xg for 2 min.) prior to electrophorectic seperation.

The second type of experiment was to incubate transfers from protein gels with radiolabelled 5S RNA or tRNA. The rationale is that renaturing protein molecules might regain specificity for RNA binding (85). Nonspecific binding is blocked by adding excess amounts of other RNA species in a non-labelled form. The results of this experiment are shown in Fig.20. Using 5S RNA as the probe, all of the proteins already implicated in 5S RNA metabolism are identified by this procedure, although the protein of Mr 28000 (P28) which anti-P43 recognises is evident only in the total oocyte protein and may have relatively little binding capacity. The only addition is a group of ribosomal proteins in the range Mr 10000-20000. Thus, there is no evidence for a large repertoire of potential 5S RNA-binding proteins.

Fig.20. Binding of radiolabelled 5S RNA to proteins from fractions of homogenized oocytes. 600 stage 2 oocytes were homogenized and one third of the homogenate was used for total protein (lane 1). The remainder of the homogenate was seperated into centrifugation fractions for protein electrophoresis as follows: (lanes2 and 3) pellets 2 and 3 as defined in Section 1:4; (lane 4) 80S ribosomes; (lane 5) 42S particles; (lane 6) 7S particles; (lane 7) supernatant. After transfer of proteins to nitrocellulose, the transfer was reacted with ^{125}I-labelled 5S RNA (0.1ug, ~4x10 cpm) for 1hr then wahed three times before an autoradiograph was produced with a three day exposure. Mr values (times 10^{-3}) for labelled bands corresponding to proteins described in the text are indicated. Each lane contains the amount of protein present in 200 oocytes from the fractions taken.


1 2 3 4 5 6 7
48-
43-
40-
33-
28-
17-

Using tRNA as the labelled probe, both P43 and P48 bind the RNA to some extent (P43 is variable), consistent with the reconstitution experiments described in Section 1:19. In the 7S region of a sucrose gradient there is binding of the tRNA, this occurs with a protein of Mr 45000. This is probably not P40 (Fig.21). Binding occurs to small ribosomal proteins up to Mr 20000. Analysis of these interactions have not been carried out further.

1:28 Utilization of Storage Particle Protein in Ribosome Formation

Neither anti-P48 nor anti-P40 reacts in immunoblotting assays with any of the ribosomal proteins. However, anti-P43 binds to two polypeptides, of Mr 28000 (P28) and 17000 (P17), both contained within the material sedimenting at 80S (Fig.22A). A component of Mr 28000 is detected using anti-P43 occasionally in material sedimenting at 42S, but only at stages when the 42S storage particles are present (Fig.22B). A very similar component is detected also in the sedimentation cut taken between 100xg and 3000xg (Pellet 2) but not in the cut taken about 3000xg (Fig.23B). Since a major constituent of Pellet 2 is nucleolus, a possible explanation of these distributions is that P28 and P17 are cleavage products derived from P43, and that P28 is associated with nucleoli or with extra-nucleolar matrix as an intermediate step in the transfer of 5S RNA from 42S storage particles to ribosomes. A possibility exists that P28 may have a function in ribosomal RNA production. This is suggested by the increase in amount of P28 in pellet 2 on going from stage 1 to stages 2 and 3, and this increase compliments the timing of nucleolar development (Fig.23C).

Fig.21. Binding of radiolabelled tRNA to proteins from fractions of homogenised oocytes. Previtellogenic ovary was homogenised and separated into fractions as follows: lane 1, 7S; lane 2, 10S-25S; lane 3, 42S particles; lane 4, 42S-80S; lane 5, 80S ribosomes; lane 6, greater than 80S. After transfer of proteins to nitrocellulose, the transfer was reacted with P-labelled tRNA and autoradiograph was produced. Mr values (times 10^{-3}) for labelled bands corresponding to proteins described in the text are indicated.

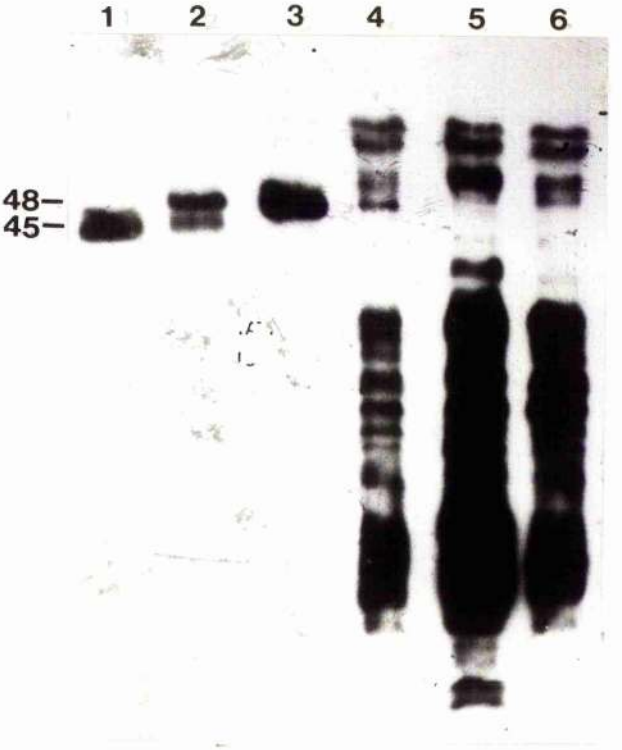
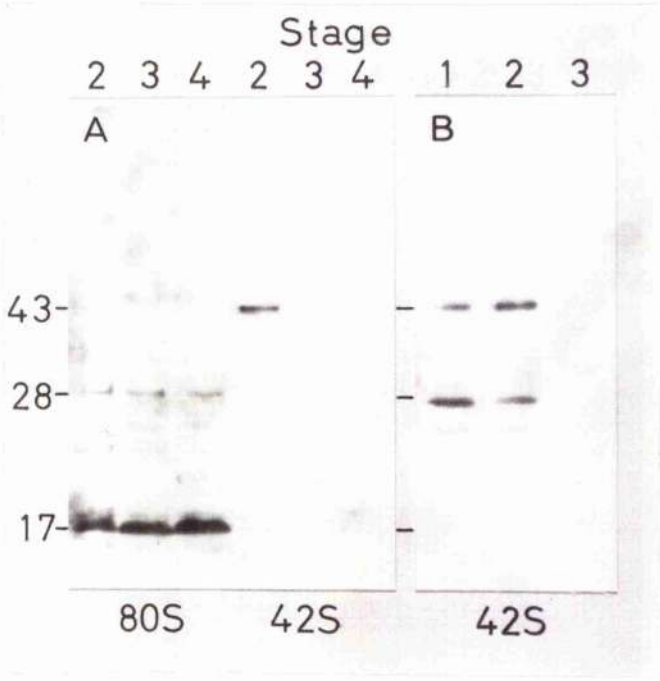

1
2
3
4
5
6
48–
45–

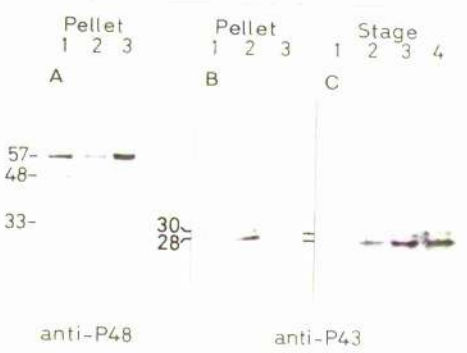
Fig.22. Immunoblotting of proteins from ribosomes and 42S particles with anti-P43. (A) Comparison of ribosomal proteins (80S) and storage particle proteins (42S) taken from the same preparations of stage 2, 3 and 4 oocytes showing cross-reacting bands at Mr 28000 and 17000. (B) The occasional occurrence of the Mr 28000 band in 42S particles from stages 1 and 2.

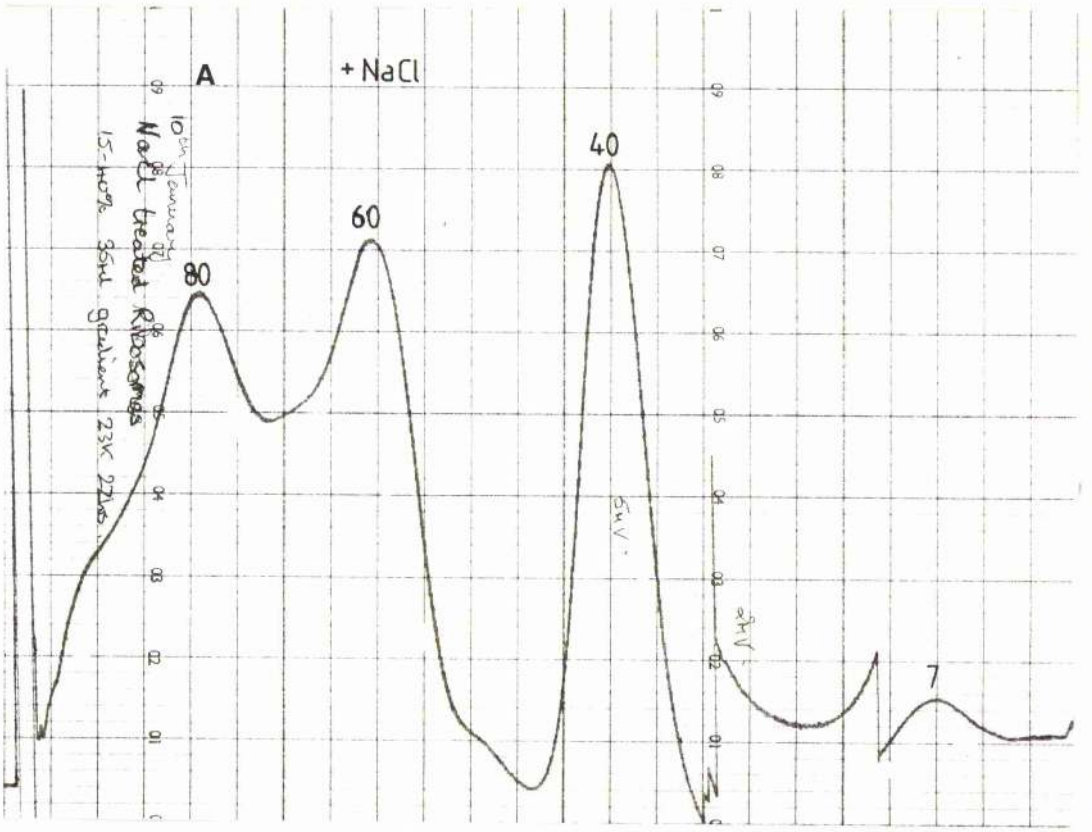

Stage
2 3 4 2 3 4 1 2 3
A
B
43-
28-
17-
80S
42S
42S

In reacting anti-P48 with the pellet fractions, a major additional component is detected with Mr 57000 (Fig.23A). The only additional information on this protein is that it is located in the 7S region in early oocytes (Fig.14) and rapidly sedimenting material. Anti-P40 reacts with none of the pellet fractions from stage 1 and 2 oocytes.

P17 has been found to be the single genuine ribosomal protein that cross-reacts with anti-P43 To investigate in which ribosomal subunit P17 was located, ribosomes were prepared from mature ovary which contained a large contribution of ribosomes from mature oocytes. These ribosomes were collected as an 80S monosome peak from a sucrose gradient, then pelleted by centrifugation at 100000xg for 2hr. The pellets were then raised in a buffer containing either 0.5M NaCl or 30mM EDTA, both the treatments causing ribosome dissociation into its constituent 40S and 60S subunits with a small amount of a 7S component (Fig.24). The proteins in these subunits were separated on an SDS-polyacrylamide gel and immunoblotted using anti-P43. Fig.25 shows that P17 is located only in the intact 80S monosome and the 60S subunit, using either treatment to separate the ribosomal subunits. Analysis of Xenopus liver ribosomal protein by immunoblotting revealed a protein of approximately Mr 17000 reacting with anti-P43 (Fig.26). This observation demonstrates that P17 is not a component peculiar to oocytes. Thus if P17 is really a cleavage product of the 5S-RNA binding protein P43 there may be a common mechanism for incorporation of 5S RNA into ribosomes of both somatic cells and oocytes. Treatment of rat liver 60S ribosomal subunits with KCl, followed by centrifugation in Cs_2SO_4 causes release of a 7S particle with a bouyant density of approximately 1.35g/cm^3 which contains 5S

Fig.23. Immunoblotting of proteins from low speed pellets with anti-P48 and anti-P43. (A) Reactions of anti-P48 with proteins contained in pellets 1, 2 and 3 produced by a series of differential centrifugation steps as described in Section 1:4. (B) as for (A) but using anti-P43. (C) Pellet2 recovered from oocyte stages 1, 2, 3 and 4 reacted with anti-P43. The Mr values (times 10^{-3}) for the labelled bands are indicated.


Pellet
1 2 3
A
Pellet
1 2 3
B
Stage
1 2 3 4
C
57-
48-
33-
30-
28-
anti-P48
anti-P43


A
+ NaCl
80
60
40
7
10th January
NaCl treated Ribosomes
15-40% 35ml gradients 23K 22hrs.

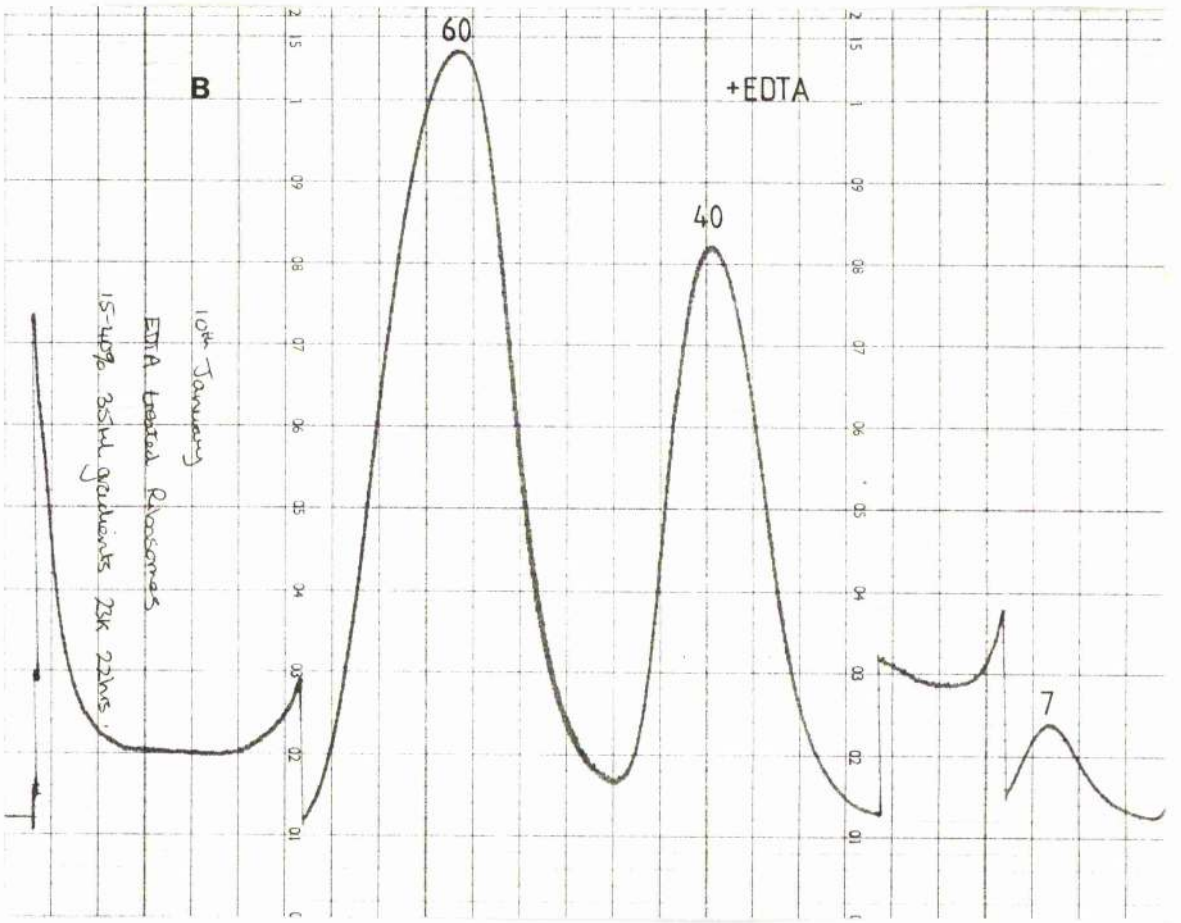

B
+EDTA
60
40
7
10th January
EDTA treated Ribosomes
15-40% 35ml gradients 23K 22hrs.

Fig.25. Immunoblotting of ribosomal subunits. Proteins in the fractions taken in Fig.24 were separated on a SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose was reacted with anti-P43 followed by ^{125}I-labelled protein-A. The anti-P43 cross-reacting 17KD band is indicated (17).

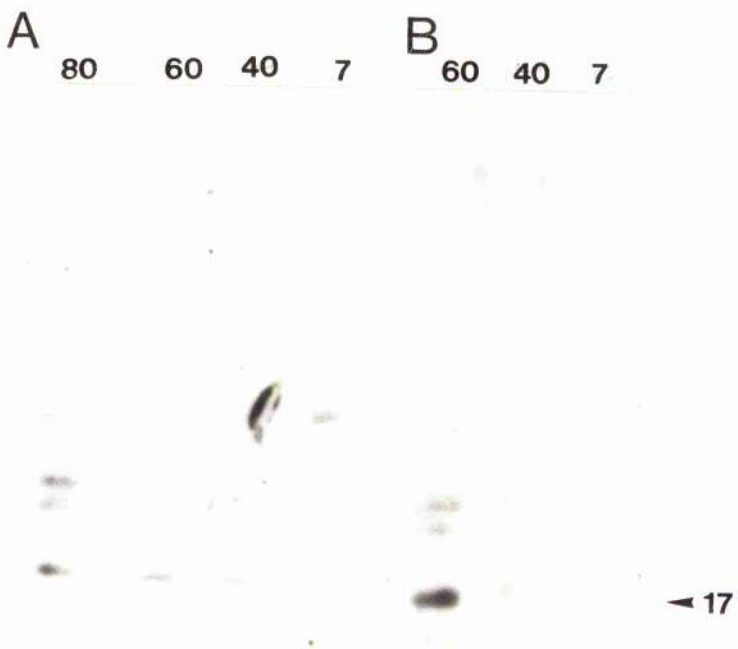
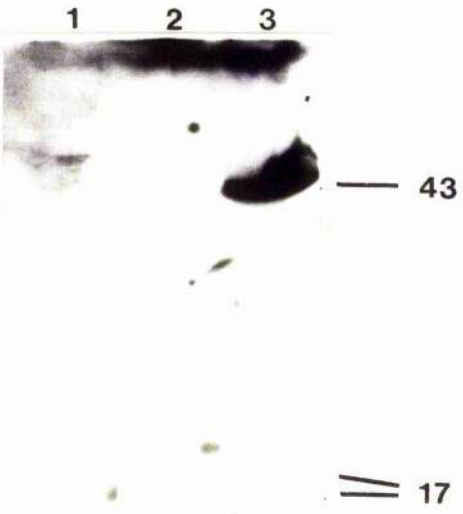

A
80
60
40
7
B
60
40
7
17

Fig.26. Cross-reacting protein in Xenopus ribosomes. Ribosomes were prepared from Xenopus oocytes and liver cells as described in Section 1:4 and 1:5. After separation of the protein components of these ribosomes by SDS-polyacrylamide gel electrophoresis, the proteins were electrotransferred to nitrocellulose. The nitrocellulose was then reacted with anti-P43 followed by ^{125}I-labelled protein-A and set up for autoradiography at -70 C. lane 1 oocyte ribosomes; lane 2, liver cell ribosomes; lane 3, 42S particles. Mr values (times 10^{-3}) are indicated.

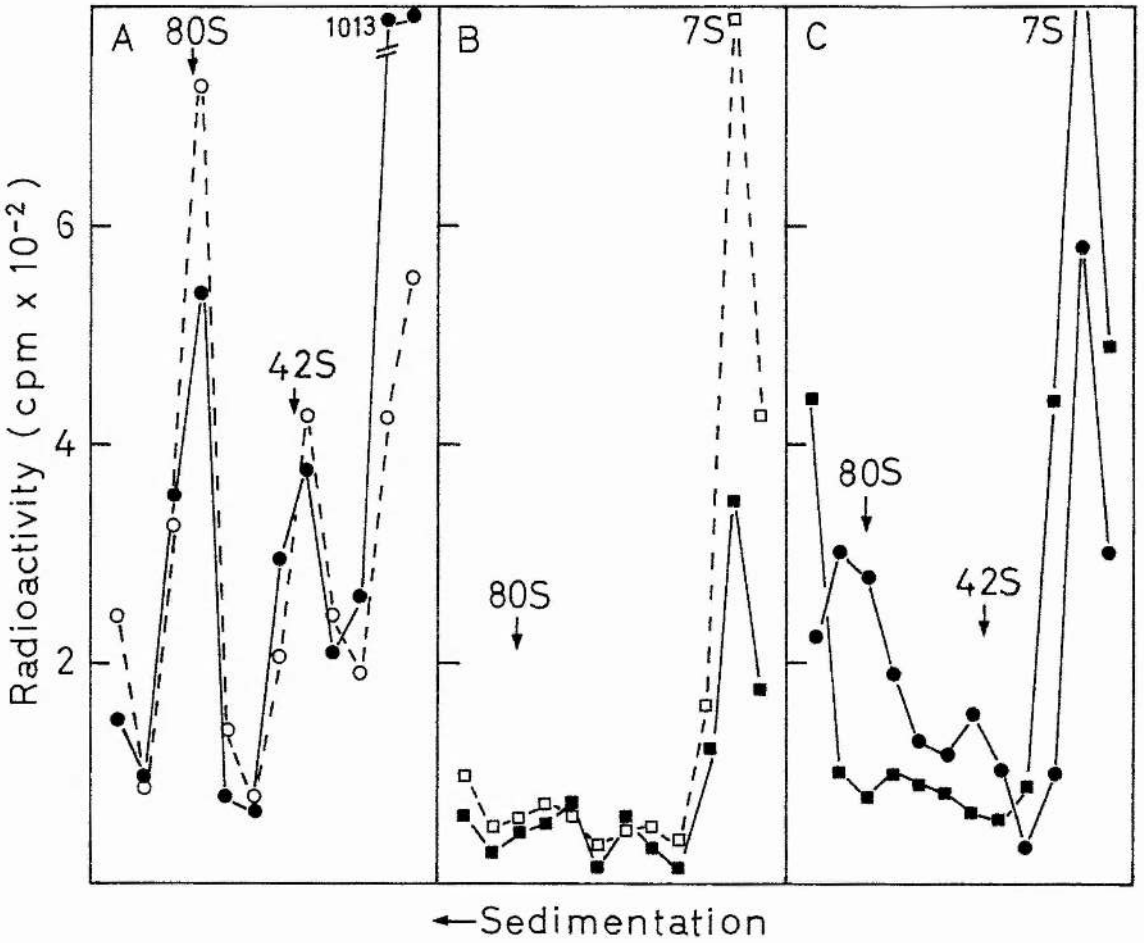

1
2
3
43
17

RNA together with the protein L5 (104). Using this analysis with oocyte 60S particles, several proteins were found in association with 5S RNA from a peak of similar density, but no significant amounts of protein at Mr 17000 were observed (data not shown). This suggests that this protein does not stay tightly bound to 5S RNA subsequent to incorporation into the 60S ribosomal subunit.

Manipulation of oocytes by microinjection has helped to gain insight into some of the control mechanisms involved in many different processes (53), including expression of exogenous genes (105) and processing of precursor tRNA molecules to a mature form (106). This technique has been applied here to follow the fate of RNP particles which are usually being utilized at the stage of oocytes injected.

42S or 7S RNP particles were labelled by incubating previtellogenic ovary in the presence of ^{14}C amino acid mix to label the proteins, or ^{3}H-uridine or ^{32}P-inorganic phosphate to label the RNA components of these RNP particles. Following concentration, the 42S or 7S particles were injected into the cytoplasm of stage 4-5 oocytes, and after a period of incubation, the utilization of the 42S or 7S particle proteins and RNA into other cellular components was analysed by fractionating an oocyte homogenate as described in section 1:4. In this analysis, labelled protein and RNA could be detected in the 42S region of the sucrose gradient after injection of labelled 42S particles; however, labelled protein and RNA could also be observed in both the 80S and 7S regions (Fig.27). From a series of experiments up to 31% of the total ^{14}C and 22% of RNA counts were incorporated into the 80S peak. Both ^{14}C and ^{3}H labels were also

Fig.27. Incorporation of ^{14}C-labelled protein (solid symbols) and ^{3}H-labelled RNA (open symbols) into ribosomes. 42S and 7S particles were labelled and isolated as described in Section 1:4. After pelleting, the 42S or 7S particles were raised in sterile distilled water and microinjected into the cytoplasm of 200 stage 4-5 oocytes. Following an incubation period of 72hr, the oocytes were homogenised and fractionated on a sucrose gradient. One-fifth of each fraction was taken and TCA precipitated. The precipitate was collected on glass fibre filters and the radioactivity determined in each fraction. The ribosome peak (80), 42S particle (42) and 7S particle peaks (7) on the sucrose gradient are indicated. (A) Incorporation of $^{14}C/^{3}H$ counts after microinjection of $^{14}C/^{3}H$ labelled 42S particles. (B) Incorporation of $^{14}C/^{3}H$ counts after microinjection of $^{14}C/^{3}H$ labelled 7S particles. (C) Comparison of ^{14}C (protein) distribution after microinjection of ^{14}C-labelled 42S and 7S particles (■ injected 7S particles, ● injected 42S particles).


A
80S
1013
42S
B
7S
80S
C
7S
80S
42S
Radioactivity (cpm x 10⁻²)
6
4
2
←Sedimentation

detected in the 7S region - up to 11% and 20% respectively. After microinjection of labelled 7S particles, few ^{14}C counts could be detected in any part of the gradient other than the 7S region. However, when the 7S particles were labelled with ^{32}P, labelling the RNA component, up to 35% of the injected counts were incorporated into the 80S monosome peak. A similar experiment using. ^{32}P-labelled 42S particles showed the presence of label in ribosomes, 42S and 7S region. RNA was extracted from the ribosomes and the 7S region and was found to be 5S RNA (Fig.28). The incorporation of ^{32}P label into cellular fractions by way of labelled protein is unlikely since the 42S and 7S particle proteins are found not to be phosphorylated in vivo (data not shown).

Attempts to identify the labelled protein component in the 80S monosome peak have not been conclusive. Using ^{35}S methionine or cysteine to label 42S particles results in poor incorporation of the label in the 80S monosome peak. However, the extracted 42S particles from the injected oocytes generally has a smaller quantity of P43, which even may be depleted entirely (Fig.29). The best results of incorporation into the 80S monosome peak occur when ^{14}C labelled 42S particles are injected into oocytes, but the specific activity of the label does not appear to be sufficient for identification of the labelled product. The amount of injected 42S particles utilized in each injection also varies in different experiments, which may indicate variability in the rate at which the oocyte is using these exogenous particles.

Fig.28. Incorporation of 5S RNA by ribosomes. 42S particles were labelled with ^{32}P-inorganic phosphate as described in Section 1:4. Labelled 42S particles were pelleted and raised in sterile distilled water and microinjected into 200 stage 4-5 oocytes. Following an incubation period of 72hr, the oocytes were homogenised and fractionated on a sucrose gradient. RNA was extracted from ribosomes (80), 42S particles (42) and 7S particles (7) then electrophoresed on a 10% polyacrylamide gel. The gel was then dried and set up for autoradiography.

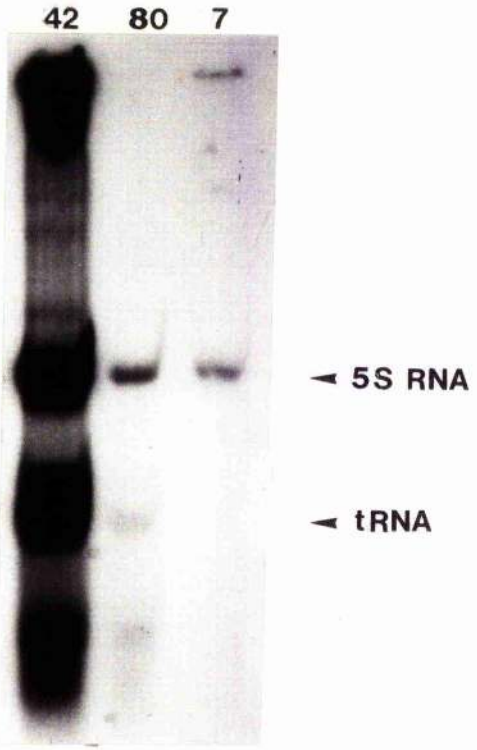
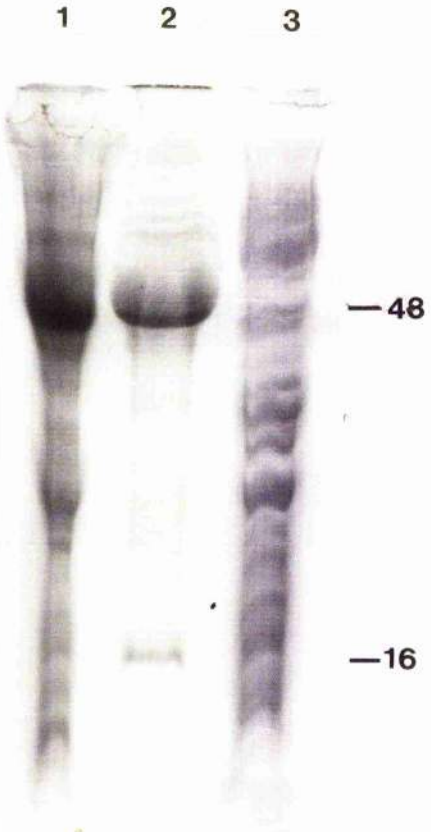

42
80
7
5S RNA
tRNA

Fig.29. Protein profile of 42S particle proteins after microinjection. 42S particles were labelled with ^{35}S-cysteine and isolated as described in Section 1:4. 42S particles were then concentrated 100-fold by dialysis against 20% polyethylene glycol in 50mM NaCl, 10mM Tris-HCl pH7.4 and 5mM 2-mercaptoethanol. The final concentration of these particles was typically 400-500mg RNP/ml equivalent to 200-250mg protein/ml (approx. 500000 cpm/ul). 200 stage 4-5 oocytes were microinjected with the labelled 42S particles in to the cytoplasm. Following a 72hr incubation the 7S particles (lane 1), 42S particles (lane 2) and 80S ribosomes (lane 3) were isolated as described in Section 1:4. Proteins from these fractions were separated on a 15% SDS-polyacrylamide gel and coomassie stained. Molecular weight of the major bands in the 42S region are indicated ($x10^{-3}$).


1
2
3
48
16

Analysis of ribosomal proteins by 2D electrophoresis (52), followed by immunoblotting with anti-P43, reveals that the cross-reaction with P17 is with one spot on the 2D gel. This cross-reacting spot, which migrates towards the basic end of the 1st dimension gel corresponds to protein L19 (52) when compared to a stained gel (Fig.30).

1:29 Identification of P43 at the nucleolus.

The nucleolus is the site of rRNA synthesis and ribosome assembly (107-109). If P43 plays a role in ribosome synthesis, the protein should be detectable in this cellular fraction. The evidence that P43 does appear at the nucleolus and indeed may be cleaved to P17 is two-fold.

First, ^{14}C labelled 42S particles were injected into the cytoplasm of stage 3 oocytes and incubated for 3-4 days at room temperature. After this period of incubation nuclei were isolated from the injected oocytes and the contents spread on glass slides. These slides were then coated with a photographic emulsion, set up for autoradiography and developed 3-4 weeks later. Fig.31 shows that the nucleoli have incorporated injected label. The distribution of label is mainly around the periphery of the nucleoli. In some preparations labelling appeared in the nuclear material distributed between nucleoli. These results taken together suggest that the oocytes have utilized the injected 42S particles and transported a 5S RNA-RNP complex back to the nucleus (nucleoli).

Fig.30. 2-dimensional separation of ribosomal proteins and localization of P17. Ribosomes were isolated as described in Section 1:4. The proteins were extracted with acetic acid and lypholyzed. Lypholyzed protein was prepared for separation on an acid-urea polyacrylamide gel in the first dimension and a SDS-polyacrylamide gel in the second dimension. (A) 2-dimensional separation of ribosomal proteins stained with silver salts. (B) 2-dimensional separation of ribosomal proteins electrotransferred to nitrocellulose and immunoblotted with anti-P43. →Indicates the postion of L19.

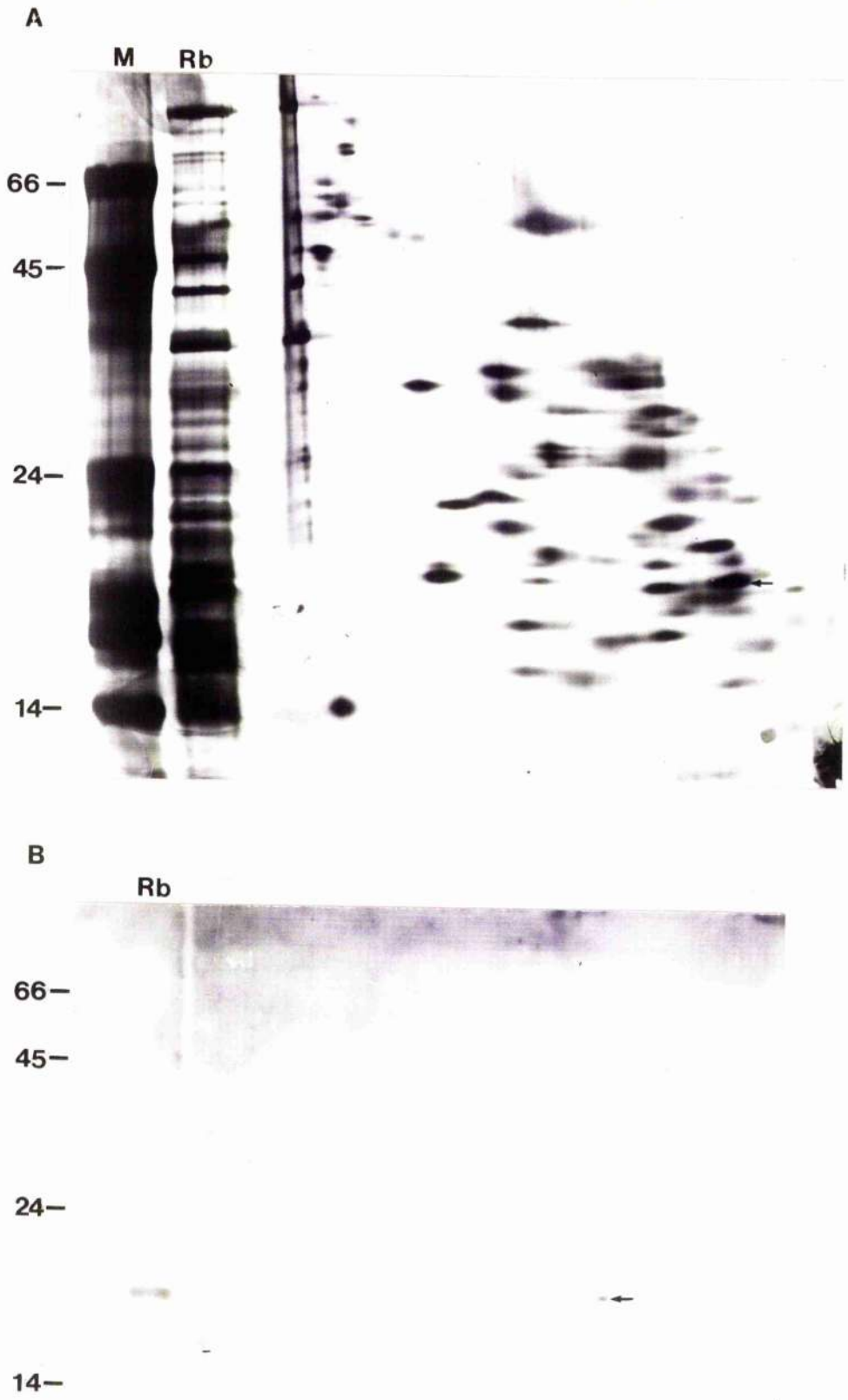
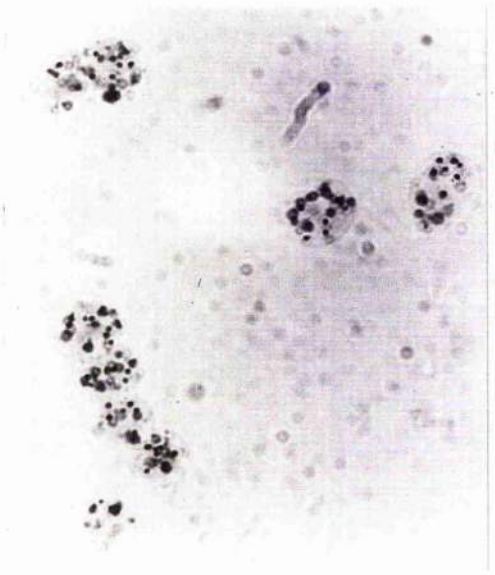

A
M
Rb
66
45
24
14
B
Rb
66
45
24
14

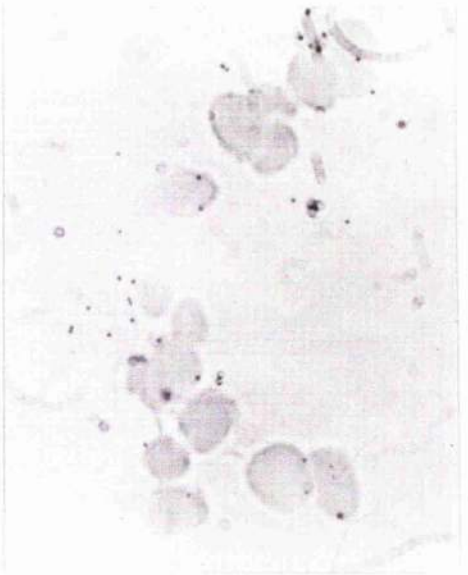
Fig.31. Incorporation of labelled 42S particle protein in nucleoli. 42S and 7S particles were labelled with ^{35}S-cysteine then microinjected into 200 stage 3-5 oocytes. Following a 90hr incubation period nucleoli were prepared as described in Section 1:18. The nucleoli were fixed to coverslips and mounted on slides. The mounted slides were dipped in fine grained nuclear emulsion and dried. Autoradiographs were exposed for 3-4 weeks at -70°C.

Microinjected:

42S RNP



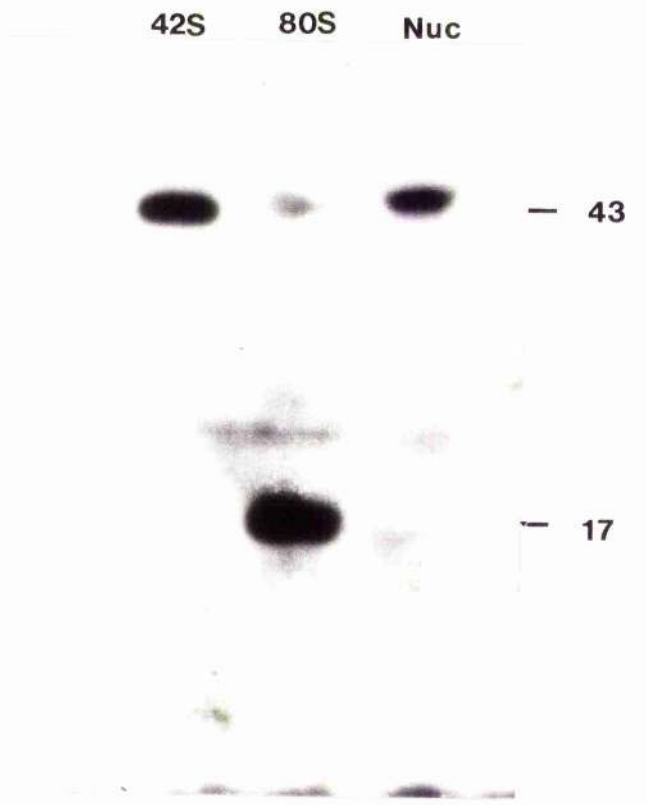
7S RNP



In contrast, the injection of ^{35}S cysteine-labelled 7S fractions does not produce labelling of the nucleoli above background. These results are consistent with immunofluorescent studies which show that anti-P43 binds to nucleoli, whereas anti-P48 or anti-P40 do not (J. Coxon pers comm.).

The interpretation that the P43 component of 42S particles is taken up by nucleoli is supported by immunofluorescent studies on cryostat sections (110) of ovary, which show immunostaining with anti-P43 of both nucleus and nucleolus. Futher support comes from experiments in which 42S particles, ribosomes and nucleoli were prepared from a mid-vitellogenic ovary. The proteins from these oocyte fractions were run out on an SDS-polyacrylamide gel, then immunoblotted with anti-P43. This antibody stained solely P43 in the 42S particles, P43 and probably P17 in the nucleolar preparation and mainly P17 in the ribosomes (Fig.32) (this confirms the presence of both P43 and P17 in the nucleolus). Anti-P48 and anti-P40 do not cross-react with any nucleolar or ribosomal protein (data not shown).

Fig.32. Immunoblotting of oocytes fractions representing different stages of 5S RNA utilization. 42S particles and ribosomes were prepared from mid-vitellogenic ovary. Crude preparation of nucleoli was obtained from 60 oocytes of the same ovary as described in Section 1:7. The proteins of each fraction was separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose. After reacting the nitrocellulose with anti-P43 followed by ^{125}I-labelled protein-A, the nitrocellulose was set up for autoradiography. (lane 1) 42S particles; (lane 2) 80S ribosomes; (lane 3) nucleoli. Mr values (times 10^{-3}) for the labelled bands are indicated.


42S
80S
Nuc
43
17

DISCUSSION

The data presented here unfolds a complex set of activities centred on 42S storage particles with respect to stabilization, distribution and utilization of their components. Particle proteins appear to have key roles in 5S RNA and tRNA transcription and in ribosome assembly.

That the components of 42S particles are organised into smaller ribonucleoprotein complexes is evident from different lines of evidence: the two major proteins of the particles, P48 and P43, have poor solubilities in 'physiological' solutions unless they are bound to either 5S RNA or tRNA; when the 42S particle naturally, or on treatment with EDTA (Fig.2) or high salt (Fig.3) breaks down, components are released only as defined sets of smaller ribonuleoprotein complexes. In its earliest developmental state (stage 1) the 42S particle is seen to contain largely P48 in combination with both 5S RNA and tRNA (Fig.14). Since this protein can bind either one molecule of 5S RNA or two to three molecules of tRNA under fairly stringent conditions of reassociation (Fig.1), it would appear that these particular interactions relate to the situation in vivo. Indeed, a P48-5S RNA complex is released from 42S particles treated with EDTA (Fig.2). P48 is also observed to interact with both 5S RNA and tRNA in filter binding experiments (Fig.20 and 21). Furthermore, density analysis of salt-dissociated 42S particles from stage 1 oocytes reveals small ribonucleoprotein complexes containing one molecule of protein in combination with two and three molecules of RNA (Fig.3B), a situation that has been found

to occur only between P48 and tRNA (Fig.1).

The in vivo situation regarding a P43-RNA association is complex. In filter binding experiments, P43 inconsistently binds tRNA (data not shown). Although P43 has been isolated as an RNP complex with 5S RNA (64) and binds 5S RNA in the filter binding experiment (Fig.20), the results in Fig.2 suggest that under certain conditions P43 can also associate with tRNA. The dynamic state of the 42S particle does not make analysis easy. Although P43 is able to bind either 5S RNA or tRNA (Fig.1), P43 cannot be found in a ribonucleoprotein complex after EDTA treatment, when 7S particles can be obtained containing P48 and 5S RNA.

P40 (TFIIIA) binding to 5S RNA has been clearly demonstrated (55). Filter binding (Fig.20) and in vitro reconstitution (Fig.1) confirm this finding and give confidence of the reliability of the methods employed.

Direct evidence is presented for changing patterns in the composition of the 42S storage particle of Xenopus oocytes. Initially the analysis was carried out using whole ovary. As Fig.4 shows, heterogeneity of protein composition exists within the 42S particles taken from one individual animal. When this analysis is extended to ovaries of differing maturity, heterogeneity can be seen to exist not only in protein composition but also in RNA content of the 42S storage particle (Fig.5).

A more detailed analysis of the composition of the storage particles within individual stages of oocytes is made possible by the use of antibodies monospecific to each of the proteins. At stage 1, P48 is already an abundant protein of the 42S particle (Fig.12A). Little change is observed in progressing to stage 2, but it declines in stage 3 when the abundance of 42S particles is also declining (figs.11 and 12). In contrast, P43 at stage 1 is not present at its maximal level, which occurs at stage 2 but is then undetectable by stage 3 (Fig.12). The absence of P43 by stage 3 seems inconsistent with the ratio of P48 to P43 in 42S particles taken from ovary containing all stages of oogenesis. A possible explanation is that P48 is broken down to P33 by proteolytic cleavage as a result of factors (enzymes?) released during the homogenization step (Fig.17), thus altering the P48 to P43 ratio in homogenates of an ovary containing all stages of oogenesis.

P40 (TFIIIA) is abundant as a 7S RNP particle during stages 1 and 2, but absent by stage 3 (Fig.12F and also (65)). These results are consistent with those of Shastry et al. (103), but differ in the timing of the reduction of P40 within the oocytes. In their analysis the level is nearly constant up to stage 4. The main consideration is the requirement for 5S RNA in ribosome assembly and from the results presented here it is not certain whether the 5S RNA in 7S storage particles or 42S storage particles is utilized first. It would appear however from Fig.12 that the decline in P40 starts before the decline in P48 or P43 at stage 2 and coincides with the onset of rRNA synthesis at the nucleoli (53). The results of Shastry et al.(103) imply that the 5S RNA of 42S particles is utilized before

that of the 7S particle. None of the immunoreactive protein which crossreacts with anti-TFIIIA (a transcriptionally inactive protein of Mr 2000 higher than TFIIIA) is found at stage 6 although its presence at this stage has been reported by other workers (103).

An unusual situation exists in very early developing ovary (taken from Xenopus only 1 month post-metamorphorsis) when individual oocytes cannot be easily distinguished. When a 42S/7S preparation is made from this tissue (figs.13 and 14) and the presence of the proteins detected by immunoblotting, P48 is seen in both the 42S and 7S region of a sucrose gradient (Fig.14). In contrast P40 and P43 are not detectable (Fig.14). Unless large amounts of P40 (and P43) are present elsewhere in the gradient or in material pelleted at 10000xg (for instance P40 may be pelleted with chromatin), one must conclude that P48 appears earlier than P40 or P43 and is capable of forming a 42S particle on its own.

About 20% of the 5S RNA is associated with neither ribosomes nor TFIIIA by the end of oogenesis (65). Here it is demonstrated that the excess 5S RNA is found as a 7S RNP particle and is associated with a protein of Mr 33000 (P33). It would appear that P33 is a cleavage product of P48. P33 accumulates in a reciprocal relationship to P48 as oogensis proceeds (Fig.12D) and can be produced from P48 by either incubating labelled 42S particles with a homogenate of stage 3 oocytes (Fig.17) or occasionally after isolation of P48 by preparative electrophoresis (Fig.16). The enzyme reponsible for cleavage may be inherent in the structure of P48 itself or may be an independent protein. However, the enzyme or its activating factor appears only in late previtellogensis (Fig.12).

The function of P33 is not known, but it could stablize 5S RNA that is excess to requirement for ribosome formation at the end of oogenesis, in preparation for initial ribosome synthsis during early embryogenesis. Alternatively the P33-5S RNA complex may be a reserve for the synthesis of new ribosomes in full grown oocytes which are required due to turnover of existing ribosomes(111,112).

The activities of P40 (TFIIIA) in regulating transcription of 5S RNA genes have been described extensively (92,95,96). However, it is difficult to detect P40 as a nuclear antigen of stages 1 or 2 oocytes in immunoblotting experiments. Futhermore, using antibodies directed against TFIIIA to immunostain sectioned previtellogenic ovary no specific nuclear staining is obtained (110). Similar results are obtained with anti-P40 (J.Sommerville, pers. comm.). It has been reported that a preparation of anti-P48 produces no staining of nuclei (110); however, the anti-P48 used in my experiments gives a slight, but significant staining of nucleoplasm in stage 1 oocytes,(cf.66). This staining could be attributable to the presence of P48 at the site of 5S RNA synthesis (cf.78). P43 is not detected as a nuclear antigen at any stage but it may be present in the nucleus at a very low level involved in the transcription of tRNA genes (see section 2 (78)). It is reasonable to conclude that the presence of TFIIIA (and P43) is transient, whereas P48 tends to accumulate in the nucleus during stages 1 and 2, possibly in association with 5S RNA genes or as a pool of free protein or complexed with newly synthesised RNA.

In the case of TFIIIA, the accumulation of this protein reflects its involvement in transcriptional activity and storage. Approximately 10^{12} molecules of TFIIIA are present during early oogenesis (103) at a time when 5S RNA synthesis is maximal and produces in the order of 10^{12} 5S RNA molecules (58). Since 50% of the 5S RNA is complexed with 42S particle protein (46,54) perhaps not all TFIIIA molecules are bound by 5S RNA. However, an over production of TFIIIA would ensure the stabilization of 5S RNA and the continued synthesis of 5S transcripts by saturation of 5S genes. The inhibitory effect of 5S RNA on 5S RNA transcription would also be diminished by over production of TFIIIA molecules (62,99).

The interaction of 5S RNA with TFIIIA may cause the apparent compartmentalization of TFIIIA to the cytoplasm (110). This may be a mechanism to prevent feedback inhibition by 5S RNA or reduce the amount of 5S RNA synthesised by restricting the entry of TFIIIA into the nucleolus. The localization of uncomplexed TFIIIA is unknown, but this protein may be bound by other components to prevent it from precipitating (58,113). The amount of TFIIIA present at stage 6 could be as high as 4ng (4.5×10^{10} molecules). Although the synthesis of new TFIIIA does not occur (93)) enough exists to ensure transcription of all 5S RNA genes. Consistent with this is the ability of nuclear extracts and microinjected nuclei to support 5S RNA synthesis from exogenous 5S RNA genes (114,115).

During maturation of an oocyte to an egg there is a further 10-20 fold decline in TFIIIA. In embryogenesis the level of TFIIIA remains approximately constant on a per embryo basis upto the swimming tadpole stage (103). At the midblastula stage, 5S RNA

synthesis resumes (115-117), but following initial activity of oocyte-type and somatic-type 5S RNA genes, selective synthesis of somatic type 5S RNA occurs (116,117). Again there must be selective sequestration of TFIIIA to prevent continued activation of oocyte type 5S RNA genes since there could be greater than 10^5 molecules of TFIIIA present per cell (103). Evidence now suggests that the selective synthesis of somatic-type 5S RNA from gastrulation is due to the greater affinity of the somatic-type 5S RNA genes to bind TFIIIA (118). An ever diminishing amount of TFIIIA preferentially binds somatic-type genes after DNA replication during embyrogenesis (119,120) and oocyte-type 5S RNA gene expression is mediated through histone binding to these sequences, in particular histone H1 (121-123).

The role of P48 or P43 in transcription of 5S RNA or tRNA genes will be discussed in Section 2. No evidence for the presence of P48 in embryogenesis or adult tissues exists and the same is true for P43. However, in this latter case a cross reacting protein of approximately Mr 17000 exists in liver cell ribosomes (Fig.26), an occurence similar to that in oocyte ribosomes where this protein has been identified as possibly being protein L19 (Fig.30). Direct evidence for the cleavage of P43 to P17 (L19) is needed (by amino acid sequencing or by detection of labelled P17 after microinjection of labelled 42S particles) to establish this point unequivocally. But the demonstration that P17 is located in the 60S subunit (Fig.25), accumulates reciprocally in relation to P43 utilization (Fig.22), is probably found together with P43 in crude preparations of nucleoli (Fig.32), and the presence of labelled material at the

nucleoli after microinjection of labelled 42S particles (Fig.31) are all consistent with the idea that P17 is a cleavage product of P43 and becomes part of the ribosome structure. No detailed studies have been performed on L19 of X.laevis ribosomes and little information is available on this protein, except that L19 can be synthesized in an in vitro translation system and incorporates ^{35}S methionine (52). The mRNA for this study was prepared from mRNP of Xenopus oocytes, suggesting that there exists in mRNP fractions a separate mRNA for L19. This mRNA could code solely for L19, or perhaps for a larger protein molecule with premature termination during translation or cleavage during or after synthesis to form L19.

A protein of Mr 28,000 cross reacting with anti-P43 is also observed in oocyte ribosome fractions. The presence of this protein in ribosomes is less consistent but it does appear in a pellet produced by a series of differential centrifugation steps (Fig.23). This pellet is the result of a 3000xg spin, the main components of which are nucleoli. This protein also occurs in reciprocal quantities to P43 (Fig.22) but it is not known whether this protein is a cleavage product of P43 or possibly a cleavage product of P43 and an intermediate to the production of P17.

The possibility exists that P17 and P28 could be entirely different gene products that happen to cross react with anti-P43. Also the presence of P43 in nucleolar fractions could be the result of the protein being associated with pre-ribosomal particles, which have been shown to contain at least one specific pre-ribosomal protein:- Ribocharin at Mr 40000 (124).

The characterization of P48, P43 and P40 is critical in establishing the roles that these proteins play in the transcription and metabolism of 5S RNA and tRNA. Using the techniques of radioimmunoassay, immunoblotting, amino acid analysis and cyanogen bromide cleavage, the three proteins associated with 5S RNA in Xenopus oocytes (P48, P43, P40) are found to be structurally distinct. This agrees with the findings that chymotrypsin digestion patterns for P43 and TFIIIA (P40) are different (62) and that antibodies directed against TFIIIA do not cross-react with 42S RNP particles (94). More recently it has been demonstrated that a preparation of anti-P48 does not cross react with other particle proteins (110). The results presented here establish that there can be no simple precursor-product relationship between any pair of proteins.

During amphibian oogenesis, 5S RNA and tRNA molecules move between various cellular compartments. For instance, 5S RNA is transported from transcription sites to nucleoplasm, then to cytoplasmic storage particles and finally to ribosome assembly sites (nucleoli). Each of these steps probably is governed by the form of the protein bound to the RNA. In principle, transfer of RNA between compartments can occur either through modification of the binding protein or exchange of binding proteins. Both processes are known to occur in previtellogenic oocytes, 5S RNA is bound to P48 as demonstrated above and to P40 in 7S RNP particles (55). During vitellogensis about 80% of the 5S RNA is transferred to the nucleoli and incorporated into ribosomes in association with a protein that is neither P48 or P40 (ie; exchange has occurred) whereas the remainder

of the 5S RNA persists as a 7S storage particle (65) in assocation with an Mr 33000 cleavage product of P48 (ie; modification by cleavage has occurred).

Although cleavage products are produced from P48 and P43, we can detect in immunoblotting experiments no higher molecular weight precursors of these proteins themselves (a cross-reacting band with anti-P48 at Mr 57000 is seen inconsistently in the pellets taken by differential centrifugation (Fig.23) and in the 7S fraction of oocyte of less than 50um in diameter, but there is no further information on this). Even analysis of homogenates of whole ovary containing early oocyte stages reveals no immunostained bands of higher molecular weight. These observations also suggest that there is no stable polyprotein precursor that contains more than one of P48, P43, and P40.

It is reasonable to suppose that P48, P43 and P40 are each encoded by separate genes which, because of their related functions, may have arisen by duplication but now diverged to an extent whereby they express proteins of distinct antigenic structure. Divergence in structure is seen also when the functionally analogous proteins from different amphibia are compared. There appears to be little antigenic relatedness between the proteins isolated from the toads (Xenopus) and the corresponding proteins isolated from newts (Triturus). However, within the genus Xenopus and within the genus Triturus, P48 and P43 appear to have diverged little in structure, both proteins being indistinguishable between species in the immunological assays performed here. This contrasts with the finding that P40 shows no antigenic relatedness between the two closely

related Xenopus species and has no detectable analogue in the genus Triturus.

The immunological distinction between P40 of X.laevis and X.borealis in antibody binding and transcription inhibition (see section 2) is particularly strange because of their identical function. It is possible that at least the RNA-binding sites are conserved structural features of these proteins. It is possible that recognition of these determinants is precluded by the use of RNP structures as antigens.

The role of two separate RNP storage forms of 5S RNA in Xenopus oocytes, with P40 in the 7S particle and with P48 (and P43 and tRNA) in the 42S particle, is not apparent. The one type of (42S) storage particle in Triturus represents a simple situation but does not exclude the presence of small amounts of a P40 analogue acting as transcription factor of 5S RNA genes in Triturus oocytes. The observed difference might simply be due to the presence of a large pool of P40 in Xenopus oocytes and of a small pool of an equivalent protein in Triturus oocytes. Nevertheless, until the overall significance of the different RNA-protein interactions are fully understood, generalizations covering various diferent organisms should be avoided.

INTRODUCTION

Amongst the genes transcribed by RNA polymerase III are those encoding all species of tRNA molecules, 5S RNA and some small viral RNAs and those containing certain middle repetitive genomic sequences.

RNA polymerase III has a Mr of 780000 and consists of 10-11 subunits. RNA polymerase III alone is insufficient to initiate transcription at the correct site from any of the genes it transcribes (125). However correct initiation of transcription requires additional components whose number and nature are just beginning to be characterised (34,57,59). Much more is known about the DNA sequence signals required for transcription, particularly in the cases of 5S RNA and tRNA genes. The development of in vitro transcription assays for these genes has made them an attractive system to study (29,106,114,115,126-137).

2:1 5S rRNA genes

5S rRNA genes of *Xenopus* consist of two multigene families, those encoding oocyte-type 5S RNA and those encoding somatic-type 5S RNA. The difference between these two types of 5S RNA is restricted to an alteration of four out of 120 bases in the coding sequence although other differences occur in the flanking sequence regions (95,138).

The 5S RNA gene is 120 nucleotides in length and is well conserved between organisms (139). In X.laevis, there are approximately 20000 copies of the oocyte-type 5S RNA genes and 400 copies of the somatic-type genes per haploid genome (138). In X.borealis, there are 9000 and 700 copies of the oocyte- and somatic-type genes respectively (138). The organisation of both sets of genes is of long contiguous DNA segments consisting of tandemly repeated units of 5S RNA coding regions separated by non-transcribed spacer DNA (140). Using 5S cRNA or cloned DNA probes specific for oocyte- or somatic-type genes, the chromosomal locations of these genes have been determined by in situ hybridisation. 5S RNA gene sequences are found at the telomeres of most, if not all, of the long arms of Xenopus chromosomes (141,142). Somatic 5S RNA gene sequences are located only on the long arm of chromosome 9, whereas oocyte 5S RNA gene sequences account for the location on all chromosomes including chromosome 9 (142).

5S RNA genes have no introns to be processed. In X.laevis and X.borealis there exists a pseudogene consisting of the first 101 nucleotides (143). It is not normally transcribed but may be when cloned pseudogenes are injected into oocyte nuclei (144).

In a series of elegant experiments by Brown and his co-workers, deletion mutants established the promoter region for 5S RNA genes to be contained within a 34bp region extending from +50bp to +83bp (relative to the beginning of the coding sequence) (19,145). However, using the technique of DNAse footprinting the region protected by the transcription factor was found to extend from +45 to +96 (97), a finding supported by point mutational analysis of the

X.laevis 5S gene promoter (20).

It has been clearly established that one factor required for 5S RNA transcription is a protein molecule of Mr 40kD, known as Transcription Factor IIIA (TFIIIA) (62,97). In addition to RNA polymerase III and TFIIIA, two other factors are required for transcription (30,34). The order of action and stability of complexes formed with these additional components is known for 5S RNA genes (59); initially TFIIIA binds to the internal control region of a 5S RNA gene, a complex which is metastable. TFIIIC binds to this complex stabilizing TFIIIA interaction on the gene and allowing TFIIIB to complete the initiation complex.

During oogenesis there is an uncoordinated synthesis of ribosomal components; 5S RNA is synthesised from early in oogensis (46) using both oocyte- and somatic-type genes, the excess product then being stored until 18S, 5.8S, 28S rRNA and other ribosomal components reach the maximum level of production (55,57). The stored 5S RNA does not exist on its own, but it can be found in association with three different proteins (see Section 1).

TFIIIA (referred to as P40 in Section 1) has been shown to be able to supplement in vitro transcription assays for the transcription of 5S RNA genes (97), and also the presence of 5S RNA molecules was able to inhibit the enhancing effect of TFIIIA. This protein therefore provides the oocyte with a feedback system for the control of 5S RNA synthesis (62).

During the development of a Xenopus embryo, the synthesis of oocyte-type 5S RNA occurs from the mid-blastula transition to the late blastula stage, but is then inactivated during gastrulation (116). The mechanism of switching has not been unambiguously established. One mechanism may be through the protein antigenically related to TFIIIA (99) found in somatic cells. However, a competition analysis was used to show that there is a fourfold difference in the ability of the somatic 5S RNA gene to bind TFIIIA over the oocyte-type 5S RNA gene. It was shown that the competitive strength was due to two base changes at residues +53 and +55 (95). This was subsequently verified and it was shown that the promoter region in the oocyte 5S RNA gene may be exposed in DNAse footprinting experiments to a greater extent than the same region in the somatic 5S RNA gene (39). The fourfold difference in binding affinities may be responsible for the differential expression of oocyte- and somatic-type 5S RNA genes. This difference in affinity is exploited following DNA replication (120). From mid-blastula stage of embryogenesis to gastrulation, there is a significant reduction in the amount of TFIIIA present in each cell (103). As a result, oocyte-type 5S RNA genes become complexed with histones, in particular the binding of histone HI seems responsible for the inaccessibility of TFIIIA to the oocyte- type 5S RNA gene sequences (123,146,147).

Apart from 5S RNA genes, all other genes transcribed by RNA polymerase III have promoters which share homology with the tRNA gene promoters, that is they are split into two intragenic regions with only limited deviation from a consensus sequence (24). Thus RNA

polymerase III genes are classified into two groups. Class 1 genes are characterised by an internal split promoter consisting of two components, box 'A' and box 'B', separated by 30 to 60 nucleotides; transcription initiates 10 to 20 nucleotides upstream from the 5' end of box 'A'. Class 2 includes only 5S RNA, where transcription occurs 50 nucleotides from the 5' end of the internal control region.

2:2 tRNA genes

In all cells there is a good correlation between the number of tRNA species and the number of codons used in mRNA, although in each cell there may be a degree of heterogeneity in distribution and relative abundance of each species (148,149). Each species is represented by genes arranged as multiple copies and showing a degree of clustering (150). In X.laevis, this clustering consists of a basic unit of DNA which is 3.18Kb long, containing two $tRNA_1^{Met}$ genes and single genes for six other tRNA species (151). This 3.18Kb unit is repeated many times (151). The chromosomal location of this repeat unit is restricted to a site near the telomere of a single chromosome (152).

The length of the genes varies according to the size of the variable arm or the presence of introns which may be transcribed then spliced out (153,154).

The localisation of the promoter for tRNA was achieved by using similar techniques to those used for the localisation of the 5S promoter, namely using deletion, insertion and base-pair substitution mutants (21,155,156). These techniques established that the tRNA

gene promoters are split into two non-contiguous elements termed box 'A' and box 'B', whose presence is essential for transcription (21,155,156). These elements are generally separated by 30 to 40 nucleotides depending on the length of the variable arm. However, longer distances between the two boxes can be created by insertion mutation. These mutants are less efficient in transcription as the distance between the two boxes increases, and after a separation of 100 bp or more, the mutant gene is no longer transcribed (21).

Comparison of the box 'A' and box 'B' sequence between tRNA genes of eukaryotes reveals a consensus sequence for the two boxes (21). Comparison with the intragenic promoter of 5S RNA genes reveals some homology and differences between the control sequences of these two classes of genes. The internal control region of the somatic 5S RNA gene of X.borealis can be internally dissected into two components and the first half of this promoter is homologous and functionally equivalent to the box 'A' component of tRNA genes (24).

Much less is known about the identity of protein factors that bind tRNA genes and influence their transcription. Although the region these factors bind has been determined by DNAse footprinting experiments (41,157,158), no large scale purification has been possible, and the best available data concerns yeast tRNA transcription factor which sediments as a large macromolecule in glycerol gradients (Mr 300000) (159), but whose composition may be of several protein subunits (41).

Both 5S RNA and tRNA transcription factors remain stably bound to the intragenic promoter for several rounds or transcription. This was shown using competition experiments (121,160). However, the formation of a stable complex with some tRNA genes required two components (160), whereas with 5S RNA genes TFIIIA can form a stable complex with the DNA in the absence of any other factors (59).

2:3 Ribosomal RNA genes

In contrast to both RNA polymerase II and III, RNA polymerase I has only one type of transcription unit, namely the ribosomal RNA (rRNA) gene (161).

The organisation of these genes is of a transcription unit containing the coding sequence for 18S, 5.8S and 28S rRNA, also including internal transcribed spacer, all of which is preceeded by a spacer region containing sequences that are not transcribed (the non-transcribed spacer), and also those that are transcribed (external transcribed spacer) (33,162,163). This multigene family is tandemly repeated in a eukaryotic genome (107).

The genes are transcribed from chromosomal locations (nucleolus organizers) develop into distinct structures, nucleoli, in which not only rRNA genes are transcribed, but ribosome assembly takes place (108,109,161,164).

Again, in contrast to the genes transcribed by RNA polymerase II and III, promoters for these genes are not obvious from an analysis of the DNA sequence. There appears to be little homology between organisms, but however, within related groups homologies do exist

(9); for example, mouse, rat and human genes show strong homology from -1 to +18 (relative to the transcription start site +1) (165); X.laevis, X.clivii and X.borealis from -9 to +14 (33,166,167); and Saccharomyces rosei and S-carlsbergensis from -9 to +64 and +76 to +125 (168). Also, deletion mutants have established the requirement for sequences located immediately upstream from the initiation site (10,11,13). Base mutational analysis has established that a G residue at position -16 is extremely important for transcription from the mammalian rDNA promoter and is probably one of the key residues that interacts with the rRNA transcription factors (12).

The X.laevis promoter extends from -142 to +6 (14,169,170), but transcription is enhanced by sequences lying further upstream in the spacer region (15). The spacer effect could be attributed to sequence elements which are 60 or 81bp long which in either orientation can exert an influence and are present as multiple copies in the spacer (171). The larger the spacer preceeding the promoter region the greater the competition strength of the promoter but as a consequence the total amount of transcription decreases (171). This effect is probably mediated through the longer spacers binding more of a limiting amount of transcription factor and probably explains nucleolar dominance in X.laevis/X.borealis crosses where only X.laevis rRNA genes, which have larger spacers, are transcribed (172,173).

Little is known about the proteins involved in the regulation of the rRNA genes execpt that at least one of the transcription factors is species specific, ie. mouse rDNA is only transcribed by cell extracts of the same species. Likewise the same was concluded for

protozoan and human rRNA genes (11). The cell extracts have been fractionated chromatographically into as many as four fractions A, B, C, D, and species specificity could be attributed to fraction D which was required for faithful initiation of transcription (42).

With the background of the above information and the results of the preceeding section, the aim of the experiments described in this section were to establish the role, if any, of P48, P43 or P40 in controlling gene expression at the level of direct interaction with 5S RNA genes or tRNA genes or indirectly through the interaction with other proteins.

MATERIALS AND METHODS

2:4 Materials

Restriction endonucleases BamHI, HindIII and EcoRI were purchased from Northern Biochemical Laboratories; BglII, T4 polynucleotide kinase were purchased from Amersham International, NsiI was from New England Biolabs. Bacterial alkaline phosphatase was from Sigma Chemical Co. For Nick translation, the Amersham Nick Translation Kit was used. α^{32}P-ATP and γ^{32}P-ATP (3000 Ci/mmol) were from Amersham International. α^{32}P-dCTP (6000 Ci/mmol) was purchased from New England Nuclear.

2:5 Source of Plasmid DNAs

Plasmids pXlt81 and pJ52 containing the $tRNA_1^{Met}$ and $tRNA^{Phe}$ genes respectively were supplied by Dr Stuart Clarkson, University of Geneva. These genes were isolated from the 3.18kb repeat of X.laevis DNA which has clustered within this region the genes encoding $tRNA^{Phe}$ $tRNA^{Tyr}$, $tRNA_1^{Met}$, $tRNA^{Asn}$, $tRNA^{Ala}$, $tRNA^{Leu}$ and $tRNA^{Lys}$ (151,152,174)

Plasmids pXlo8 and pXlo31 containing multiple and single repeating units of X.laevis oocyte-type 5S RNA genes have been described (175,176). These plasmids, together with plasmid pXlor101 containing a single repeating unit of X.laevis ribosomal DNA (15) were supplied by Dr Adrian Bird, University of Edinburgh.

2:6 Nitrocellulose Binding Assay

Initial binding studies were carried out with nicked translated plasmids. 1μg of pXlo8, pXlt81 or pXlor101 were nick translated using the nick translation kit of Amersham International plus 50μCi α-^{32}P-dCTP.

Following nick translation, the plasmid DNAs were ethanol precipitated with 3 volumes of ethanol at -20°C overnight. The DNAs were collected by centrifugation at 10000xg for 30mins, vacuum dried and raised in 500μl of standard binding buffer (SBB) containing 50mM NaCl, 10mM Tris-HCl, pH 7.0, 1mM EDTA, 0.2% bovine serum albumin, 0.2% Ficoll and 0.2% polyvinyl pyrollidone.

The plasmids were sheared by sonication using a Branson sonicator at setting 6 with 6x10sec bursts. Subsequent analyses were carried out using restricted and end- labelled DNA fragments of three plasmids: pXlo8 (5S DNA) was cleaved with BamHI and/or HindIII, pJ52 (tDNA^{Phe}) was cleaved with EcoRI and HindIII and pXlor101 (rDNA) was cleaved with EcoRI and BamHl. Double digests using two restrictions endonucleases were routinely carried out in EcoRI buffer (50mM NaCl, 100mM Tris pH7.5, 10mM $MgCl_2$) in a reaction volume of 50ul for 1hr at 37°C using 10units/μg of plasmid DNA. Following restriction, 70μl of 0.3M sodium acetate pH7.4 was added followed by 120μl of cold phenol. After vortexing and spinning in an MSE Centaur centrifuge for 4mins, at the higher speed setting, 100μl of the aqueous phase was removed. 100μl of 0.3M sodium acetate pH7.4 was added to the phenol phase, vortexed and spun again. 100μl of the aqueous phase was removed and pooled with the previous 100μl of the aqueous phase. DNA fragments

were then precipitated with 600µl of cold ethanol at -70°C for 1hr or on cardice in methanol for 30mins. Precipitated fragments were then spun down in the MSE Centaur centrifuge at 11000xg for 5mins. The ethanol was removed and, cold 80% ethanol was added to rinse the pellet. This was spun for 1min in the Centaur centrifuge at 11000xg and the ethanol removed. The pellet was dissolved in 200µl of 0.3M sodium acetate pH7.4 and reprecipitated with 600µl of cold absolute alcohol. (Double precipitation ensures all phenol is removed before the addition of the next enzyme). Following the second precipitation, the fragments were spun again for 5mins at 11000xg and the ethanol removed. The fragments were then vacuum dried. The next stage was 5' dephosphorylation of the fragments: The dried precipitates were raised in 50µl of bacterial alkaline phosphate buffer (BAP buffer) containing 0.1M glycine, 1mM $MgCl_2$ pH9.4, and vortexed for 2mins. Then 0.1units of bacterial alkaline phosphatase was added per 1µg of plasmid DNA. This was incubated at 37°C for 45mins. Following this step, the fragments were phenol extracted and precipitated as above. After drying, the fragments were then raised in 50µl of T4 polynucleotide kinase buffer (containing 50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 10mM 2-mercarptoethanol) and vortexed for 2mins. 50uCi of γ^{32}P-ATP was added plus 10u of T4 polynucleotide kinase per reaction.

If all restriction fragments were to be used in the assay, then following labelling, they were phenol extracted as above. After drying, the precipitate was raised in 1ml of SBB.

If only gene insert sequences were used, they were purified by gel electrophoresis. An equal volume of FDM buffer (10% Ficoll, 1mM EDTA, 0.05 xylene cyanol and 0.05% bromophenol blue) was added to the reaction and the mixture applied to an 8% polyacrylamide gel in 80mM Tris-borate pH8.3, 1mM EDTA. Electrophoresis was for 2-2.5hrs at 15W. The labelled band(s) containing the appropriate sequence was located by autoradiography for 30-60min at 4°C. The band was excised and immersed in 1ml of elution buffer (0.5M ammonium acetate, 1mM EDTA pH7.0). Elution was for 18 hr at 37°C. This was followed by phenol extraction. 1-2µg of E.coli DNA and 10µg of bovine serum albumin was added to the aqueous phase after phenol extraction, and this phase was ethanol precipitated at -20°C overnight. Following precipitation, the fragments were spun down at 11000xg for 30min and vacuum dried. The dried fragments were then raised in 1ml of standard binding buffer.

Protein fractions to be tested for DNA binding activity were prepared for gel electrophoresis by ethanol precipitation from a sucrose gradient fraction containing the appropriate proteins, then raised in electrophoresis sample buffer. The proteins were then electrophoresed on a SDS 15% polyacrylamide gel (see Section 1). The current was stopped as the bromophenol blue front reached the end of the gel. The proteins were then electrophorectically transferred to nitrocellulose in 25mM Tris, 192mM glycine, 20% methanol pH8.3 for 20hr at 30V, 0.1A.

After transfer, the nitrocellulose filter was first washed in renaturation buffer containing 4M urea, 50mM NaCl, 0.1mM dithiothretiol, 1mM EDTA and 10mM Tris-HCl pH7.0, for 1hr. The filter was then washed three times, 20min each wash in standard binding buffer. The filter was then sealed in a plastic bag with standard binding buffer containing 250μg/ml of sonicated E.coli DNA to act as blocking agent for non-specific binding. This incubation was for 1hr and was followed by the addition of 0.2-0.25μg of restricted end-labelled or nick-translated sheared DNA or 15ng of end-labelled gene fragment to the blocking solution in the bag. This was incubated for a further 1hr before washing three times in standard binding buffer. The filter was then blot-dried between two pieces of tissue paper and set up for autoradiography for 2-18hr at -70°C.

In experiments where the proteins were incubated in the presence of zinc, 15uM $ZnSO_4$ replaced 1mM EDTA.

For the isolation of regions containing 'A' box and 'B' box sequences from plasmid pJ52, triple digestions of this plasmid were carried out using EcoRI, HindIII and BglII, all of which were active in EcoRI buffer. Fragments of 100bp and 169bp were isolated from an 8% gel as described above.

2:7 End labelling the 320bp fragment from pXlt81 containing the $tRNA_i^{Met}$ gene.

pXlt81 was restricted with NsiI to release a 320bp fragment containing the $tRNA_i^{Met}$ gene and 5' and 3' flanking sequences.

Labelling was carried out at the NsiI restricted end by removing nucleotides (at 37°C, 1hr) and filling-in with the Klenow fragment of DNA polymerase1 (at 15°C for 15mins) in the presence of α^{32}P-dATP and cold dCTP, dGTP and dTTP. The labelled DNA was then seperated on a 6% polyacrylamide gel and eluted.

2:8 Gel retention assay

Restricted end-labelled pJ52 (described above), the 269bp EcoRI/HindIII insert of pJ52, the 320bp NsiI fragment of pXlt81 or the232bp EcoRI/PvuII fragment of pUC9 (gift of L.Clarke) were used in this assay. 0.5-2.5µg of 42S particles were mixed with 2ng of restricted end labelled pJ52 in the presence of 0.1 units of RNAse A T_1 and T_2, 1µg BSA and 1µg pBR322. Binding occurred in 50-500mM NaCl, 10mM Tris-HCl pH7.4, 15uM $ZnSO_4$ (NTZ buffer), in a reaction volume of 15µl. After 30min for equilibration either at 0°C or 20°C, the reaction was diluted with 45µl of NTZ buffer at the appropriate NaCl concentration containing 5% glycerol and 0.1% Xylene cyanol. This buffer was added at the incubation temperature of the reaction. Samples were applied to a 6% polyacrylamide gel in 0.08M Tris-borate pH8.3, 1mM EDTA and electrophoresed at 15W for 2hr (177,178). The gel was then mounted on Whatmann No.1 paper, covered in cling film and set up for autoradiography for 18hr at 4°C.

2:9 Immunoprecipitation of protein-DNA complexes

Antibodies were coupled to protein A-Sepharose beads as described in Section 1:17

In vitro binding was carried out as described above, either with or without 8M urea to solubilize the components of the 42S particle. After dialyzing out the urea, the product of the binding reaction was incubated with an antibody-linked protein A-sepharose beads for 1hr at room temperature (20°C) with frequent agitation. The supernatant was then removed and the beads washed five times with NTZ buffer of the appropriate NaCl concentration. If no urea was used in the first instance, the antibody-linked beads were added directly to the binding reaction after 30min.

To release the bound DNA from the antibody-linked beads, the beads were treated with a 100µl of preincubated proteinase K at 100µg/ml in 1% Sarkosyl, 20mM EDTA, 50mM Tris-HCl pH8.4 and the protein was digested overnight at 4°C. This was followed by the addition of 10µg of yeast tRNA as carrier, then the DNA fragments were phenol extracted and ethanol precipitated at -20°C overnight. The DNA fragments were then prepared for electrophoresis by spinning down the precipitate, vacuum drying and raising in 10µl of FDM buffer (10% Ficoll, 10mM EDTA, 0.09% Xylene cyanol, 0.01% bromophenol blue). The immunoprecipitated DNA was then applied to an 8% polyacrylamide gel in 0.08M Tris-borate pH8.3, 1mM EDTA and run at 15W for 2.5hr. The gel was dried down and set up for autoradiography for 3-4 days at -70°C.

2:10 DNAse protection experiments

The EcoRI/HindIII fragment containing the 269bp insert of pJ52 was labelled at the 5' end of either the non-coding or coding strand. To label the non-coding strand the plasmid was first digested with EcoRI, end- labelled as described above and then cleaved with HindIII. To label the coding strand the enzyme digestions were reversed. The fragments were purified on an 8% polyacrylamide gel in 80mM Tris-borate, pH8.3, 1mM EDTA as desribed above. The eluted fragments were then phenol extracted and 1µg of pBR322 and 10µg of bovine serum albumin were added to act as carriers for precipitation with 3 volumes of ethanol at -20°C overnight.

Following precipitation, the DNA fragments were spun down, vacuum dried and raised in binding buffer containing 100mM NaCl, 10mM Tris-HCl pH7.4, 0.5mM $CaCl_2$, 5mM $MgCl_2$, 7.5mM $(NH_4)_2 SO_4$ and 0.1mM $ZnSO_4$. Binding was carried out in this buffer as described in Section 2:8 with either the fragment labelled on the coding or non-coding strand. After binding, 2.5-100ng of DNAse1 was added in a 50ul of binding buffer and digestion allowed to proceed for 30sec - 5min. The DNAse1 digestion was terminated with 50ul stop buffer containing 3M ammonium acetate, 0.2M EDTA and 150µg/ml of sonicated E.coli DNA. The digested DNA was phenol extracted and precipitated with 3 volumes of cold 100% ethanol at -70°C overnight.

DNA fragments were spun down, vacuum dried and raised in 5-10µl of 98% de-ionized formamide, 10mM EDTA. The DNA was denatured by heating at 90°C for 3 min and applied to an 8% DNA sequencing gel run

in 90mM Tris-borate, 2mM EDTA, pre-run for 1hr at 1200V, 50W. Electrophoresis was carried out at the above voltage and wattage for a further 2.5-3hr. Autoradiographic exposures of wet gels were made at -70°C for 1-2 days.

RESULTS

2:11 Plasmids

(a) pXlt81 contains a X.laevis tRNA. gene$^{Met}_{1}$ A 775bp fragment of the 3.18Kb repeating unit of tRNA genes was cloned into the EcoRI site of pBR322. The 'A' and 'B' boxes are shown in black (fig.33, S.G.Clarkson pers. comm.).

(b) pJ52 contains a X.laevis tRNA^{Phe} gene. A 269bp fragment of the 3.18Kb repeating unit was cloned into the Cla1/EcoRI site of pBR322. The 'A' and 'B' boxes are shown in black (fig.33, S.G.Clarkson pers. comm.)

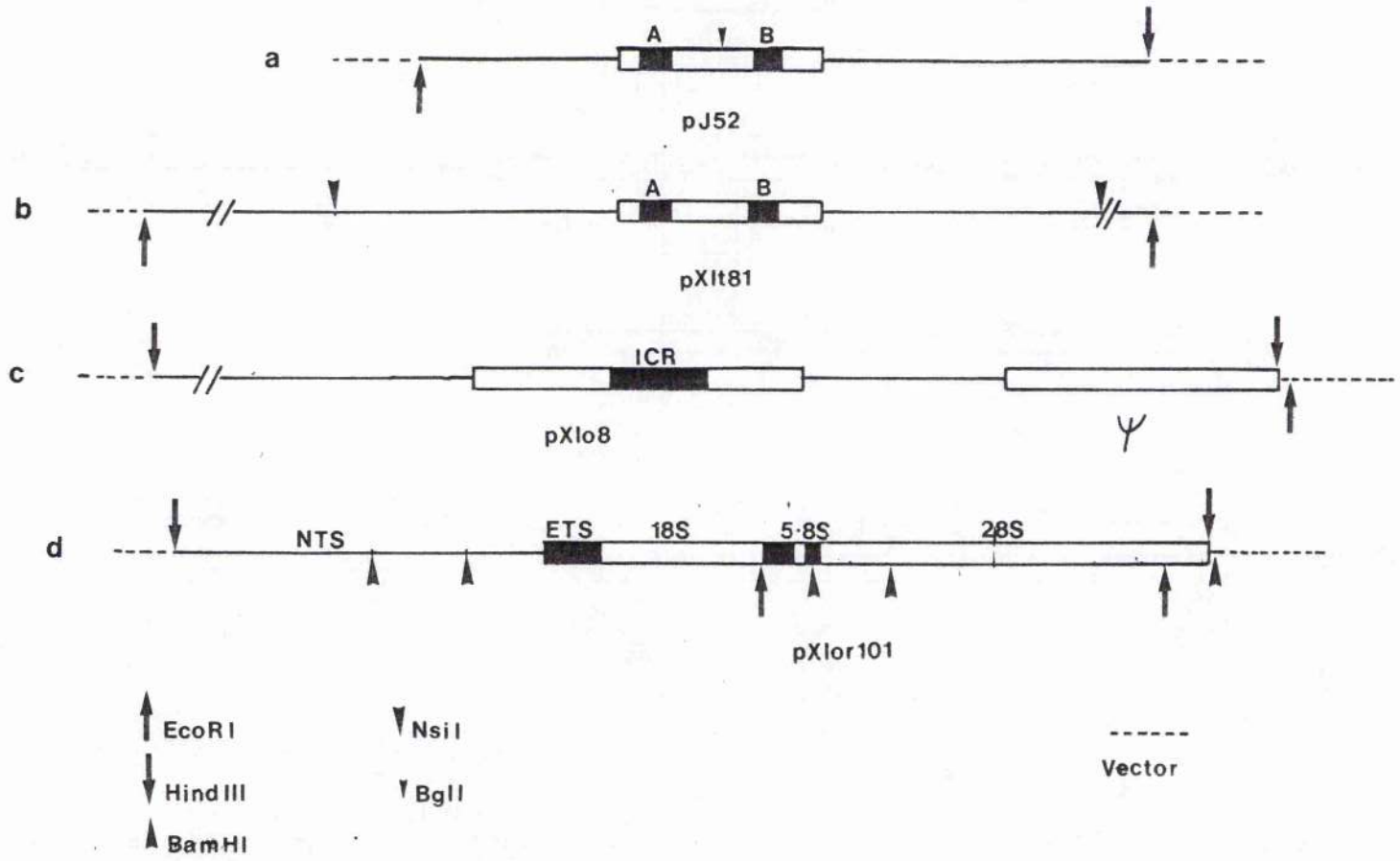
(c) pXlo8 contains a X.laevis oocyte-type 5S RNA gene. Four tandem repeating units were cloned into the Hind III site of pMB9 (115). The repeating units are 600bp. The internal control region is shown in black (fig.33).

(d) pXlor101 contains X.laevis ribosomal RNA genes. A 11.52Kb, HindIII fragment was cloned into the HindIII site of pMB9 (15, R.Reeder pers. comm.). This clone contains the non-transcribed spacer (NTS), the external transcribed spacer (ETS), 18S, 5.8S, internal transcribed spacer and 28S gene sequences (fig.33).

2:12 In vivo inhibition of 5S RNA and tRNA synthesis.

Microinjection into Xenopus oocytes of exogenous genes cloned in plasmid vectors has been a useful method of studying gene expression (105,115). This approach is used here to assess the

Fig.33. Restriction maps of the plasmids used in this thesis. (a) pJ52 containing the gene for $tRNA^{Phe}$; (b) pXlt81 containing the gene for $tRNA_1^{Met}$; (c) pXlo8 containing four tandem repeats of the 5S RNA gene (ψ is the 5S RNA pseudogene); (d) pXlor101 containing the ribosomal RNA gene transcription unit consisting of 18S, 5.8S and 28S rRNA genes.


a
A
B
pJ52
b
A
B
pXlt81
c
ICR
pXlo8
Ψ
d
NTS
ETS
18S
5·8S
28S
pXlor101
EcoR I
Hind III
BamHI
Nsi I
Bgl I
Vector

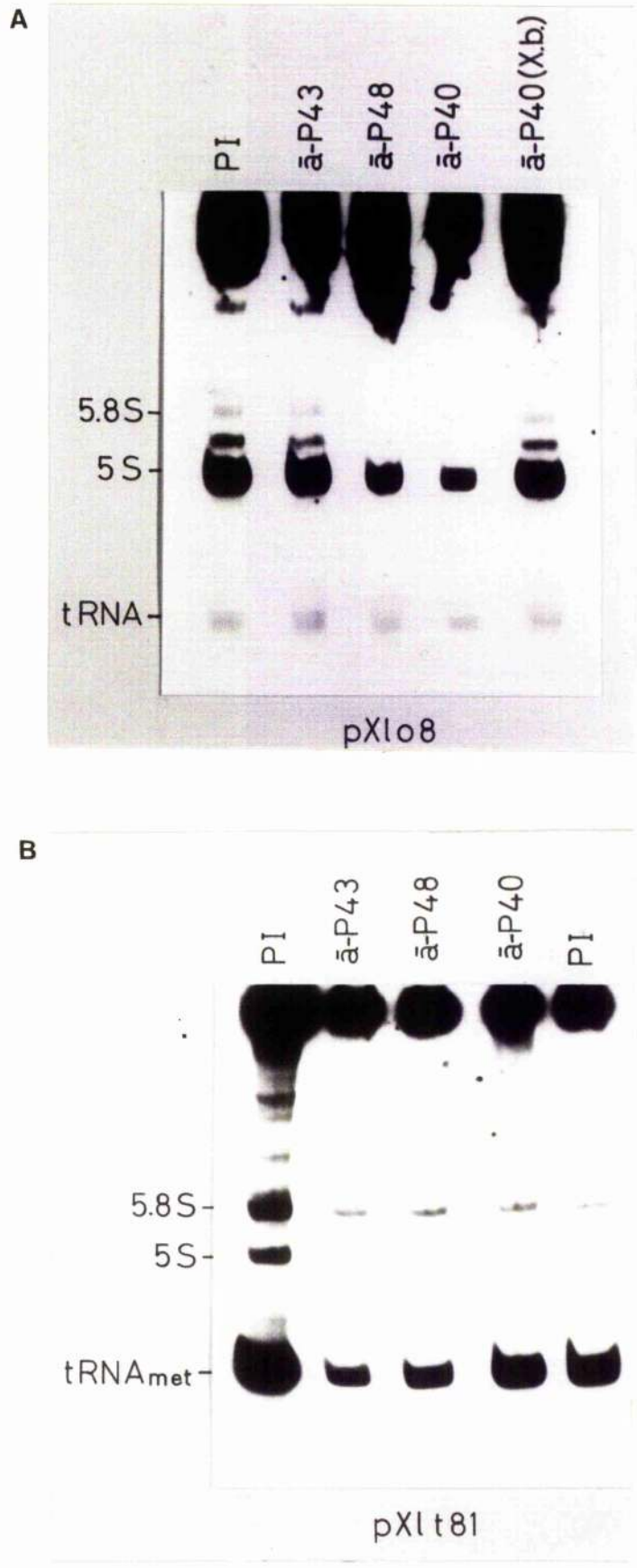
effect of antibodies raised to P48, P43 and P40 of X.laevis and P40 of X.borealis (see Section 1) on 5S RNA and tRNA synthesis from the plasmids pXlo8 and pXlt81.

To assess the effect of these antibodies on 5S RNA or tRNA transcription, exogenous 5S RNA and tRNA genes were used because the level of synthesis from endogenous genes is not sufficient for rapid analysis, and possibly preformed protein/DNA interactions may not be interrupted or dissociated by subsequently injected antibody.

(a) Inhibition of 5S RNA transcription. pXlo8, IgG and. α^{32}P-GTP were simultaneously injected into the nuclei of stage 2-3 oocytes of X.laevis. RNA extracted from oocytes injected with pre-immune IgG and followed by a 18hr incubation, shows a strong signal of ^{32}P labelled 5S RNA (fig.34). A signal of equal intensity is also obtained by injecting anti-P43 with this plasmid. However, anti-P40 has an inhibitory effect on 5S RNA transcription in this system, reducing transcription from pXlo8 by 4-5 fold in comparison with the pre-immune IgG. Moreover, anti-P48 injected with pXlo8 also reduces transcription of 5S RNA by approximately 4 fold.

The other antibody tested in this system, anti-P40 of X.borealis, does not inhibit 5S transcription from pXlo8. This is consistent with the lack of cross-reaction of antibodies raised against X.laevis P40 and X.borealis P40 described in Section 1. Injection of these antibodies does not have a general effect on reducing RNA transcription. As an internal control, the level of endogenous tRNA synthesis is the same in all of these injections.

Fig.34. In vivo inhibition of transcription. (a) inhibition of 5S RNA transcription from pXlo8. 20nl of a mixture containing 160µg/ml pXlo8 and 5.5mg/ml of antibody was injected into the nucleus of 50 stage 2-3 oocytes. The injection mixture contained either pre-immune IgG (pI), anti-P40 IgG (a-P40), anti-P48 IgG (a-P48), anti-P43 (a-P43) or X.borealis anti-P40 IgG (a-P40 X.b.). (b) inhibition of tRNA transcription from pXlt81. 20nl of a mixture containing 160µg/ml of pXlt81 and 5.5mg/ml of antibody was injected into the nucleus of 50 stage 2-3 X.laevis oocytes. The the injection mixture contained either pre-immune IgG (pI), anti-P48 IgG (a-P48), anti-P43 IgG (a-P43) or anti-P40 IgG (a-P40). The positions of 5.8S, 5S and tRNA are indicated.


A
PI
ā-P43
ā-P48
ā-P40
ā-P40(X.b.)
5.8S
5S
tRNA
pXlo8
B
PI
ā-P43
ā-P48
ā-P40
PI
5.8S
5S
tRNAmet
pXlt81

Thus the inhibitory effects of anti-P40 and anti-P48 are directed primarily against transcription of 5S RNA genes and anti-P40 shows species specificity with repect to the ability to interfere with the presumed activity of P40 (TFIIIA).

(b) tRNA transcription.

Transcription from pXlt81 was analysed by a similar protcol. In this assay a strong signal of ^{32}P-labelled tRNA was obtained from oocytes injected with pre-immune IgG (fig.34b) and also from oocytes injected with anti-P40. However, injection of either anti-P43 or anti-P48 reduced transcription from pXlt81 by 3-4 fold.

As an internal control, the synthesis of 5S and 5.8S RNA from endogenous genes is constant regardless of which antibody is injected. This indicates that the primary effect of anti-P48 and anti-P43 is directed against tRNA synthesis from pXlt81.

These results taken as a whole show that anti-P40 inhibits 5S RNA transcription (a result not unexpected from published work (62,97 103)), but has no effect on tRNA synthesis. Anti-P43 inhibits tRNA only, whereas anti-P48 has an inhibitory effect on both tRNA and 5S RNA synthesis.

The level of endogenous synthesis of 5S RNA or tRNA synthesis is relatively constant in these experiments. One interpretation is that the antibodies have less affect on RNA synthesis of preformed protein-DNA complexes.

2:13 Identification of specific 5S RNA gene and tRNA gene binding proteins.

Anti-P40 raised to the X.laevis P40 (TFIIIA) protein is monospecific and species-specific (see section 1:22). The inhibition of 5S RNA synthesis from pXlo8 by anti-P40 is probably due to the inhibition of formation of active gene complexes involving P40 (TFIIIA). The possibility therefore exists that the inhibitory effect of anti-P48 and anti-P43 on 5S RNA and/or tRNA synthesis is due to the inhibition of P48 or P43 binding to the respective gene promoters.

This possibilty is investigated here with a quick and convenient assay to detect the binding of specific DNA sequences to specific proteins. Briefly, the proteins of interest are separated by SDS-polyacrylamide gel electrophoresis, then electrophorectically transferred to nitrocellulose. The nitrocellose is processed through a series of washes which includes the incubation in radioactively labelled DNA of the sequence of interest (85).

Initially this analysis was carried out with nick-translated pXlt81, pXlo8 and pXlor101. Proteins of interest are contained in sucrose gradient fractions of ribosomes, the 42S RNP particle peak and the 7S RNP particle peak. Fig.35 shows the results of this analysis. Labelled DNA was detected by autoradiography bound to a protein of Mr 40000 from the 7S region of a sucrose gradient and to a protein of Mr 48000 from the 42S region of a sucrose gradient following an incubation with labelled pXlo8. Comparison of

Fig.35. Binding of radiolabelled DNA to fractions of homogenised oocytes. (a) previtellogenic ovary was homogenised and fractionated by centrifugation into the following fractions: 42S particles (42) and 7S particles (7). After transfer of proteins to nitrocellulose the filter was preincubated with cold, sonicated E.coli DNA (250µg/ml) for 1hr. 0.25µg of nick translated pXlo8 pXlt81 or pXlor101 was added and incubated for a further 1hr. After three washes the nitrocellulose was blot dried and set up for autoradiography at -70°C.

42S
7S
42S
7S
42S
7S
42S
7S
p48
p43
p40
pXlo8
pXlt81
pXlr101

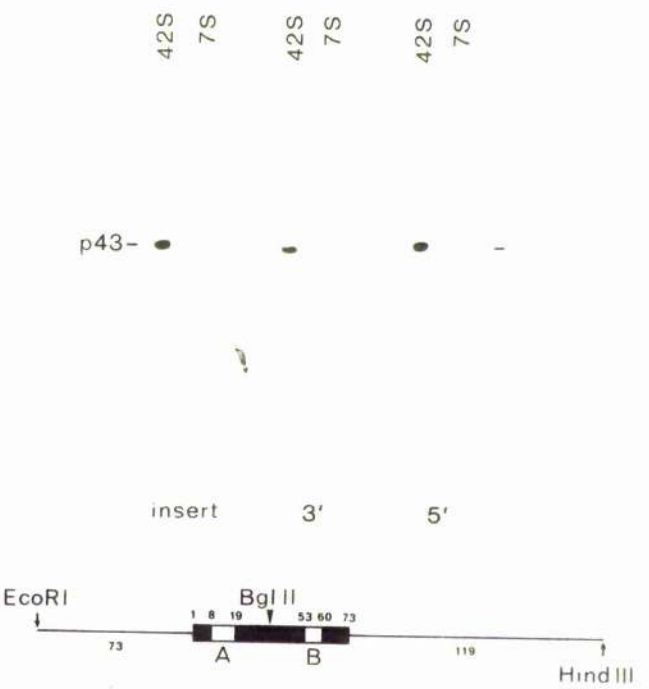
autoradiographs with Coomassie blue-stained gels gave positive identification of these proteins as P40 and P48 (fig.35).

P43 of the 42S RNP particle bound radioactive labelled DNA on incubation with ^{32}P-labelled pXlt81 and pXlor101 (fig.35). Binding to ribosomal proteins did not occur (data not shown). As reported elsewhere (113,180), zinc forms an integral part of the TFIIIA molecule (P40). However in this assay, the replacement of 0.1mM EDTA by 15μM zinc sulphate does not enhance or diminish the binding of any of these plasmids to P48, P43 or P40.

further analysis was carried out using plasmid pJ52 (containing $tRNA^{Phe}$ gene). In this analysis pJ52 was cut with EcoR1 and HindIII before 5'- end labelling. The insert was separated from the plasmid sequence by polyacrylamide gel electrophoresis, then the insert tested against 42S and 7S RNP particle proteins. P43 is the only protein to bind this insert. No binding of pBR322 to P43 was observed (data not shown).

Transcription factor binding to tRNA genes has been reported to be stronger to 'B' box sequences than at the 'A' box sequence (41,153, 157). This was tested in the binding assay using a fragment of pJ52 containing either the 5' flanking sequence plus the 'A' box sequence (-67 to +37) or the 3' flanking sequence plus the 'B' box sequence (+38 to +192). Both fragments bind to P43, indicating that they each contain sequences either within the tRNA gene or in its flanking regions which are recognised by P43 (fig.36). Plasmid pBR322 did not bind P43 in this assay (data not shown).

Fig.36. Binding of radiolabelled DNA containing either `A' box or `B' box sequences to P43. pJ52 was cleaved with EcoRI, HindIII and BglII to release the 269bp insert as two portions, one containing the `A' box (-67 to +37) or `B' box sequence (+38 to +192). The fragments were 5' end labelled with γ^{32}P-ATP and separated on a 6% polyacrylamide gel. The eluted DNA fragments were tested for binding to proteins immobilized on nitrocellulose as dscribed in Fig.35.


42S
7S
42S
7S
42S
7S
p43-
insert
3'
5'
EcoRI
BglII
1 8 19
53 60 73
73
A
B
119
HindIII

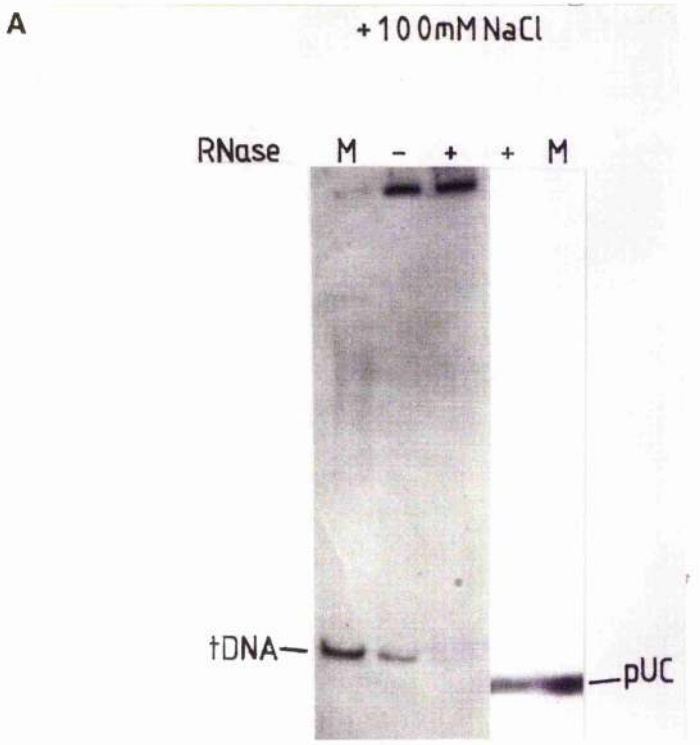
2:14 Detection of protein-DNA complexes formed between 42S RNP particles and tRNA genes.

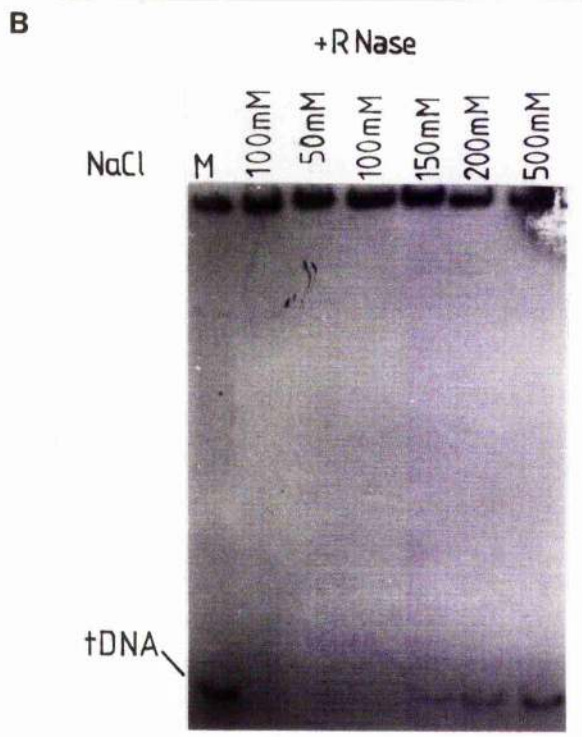
A convenient test for the formation of stable protein-DNA complexes is to mix DNA and protein in solution and then apply the material to a 6% polyacrylamide gel. Protein-DNA complexes with lifetimes of a few minutes can be detected by the gel assay, even though electrophoresis time of the analytical gel is in the order of hours (177,178). The duration of the analytical gel run has no effect on the distribution of protein-DNA complexes (177).

In this assay system uncomplexed DNA migrates to its normal position in the gel. However, DNA complexed with protein will be displaced further towards the origin depending upon the number of protein molecules bound (177,178).

The assay was used here to determine optimal conditions for binding 42S particle proteins to the 269bp insert of pJ52 carrying the $tRNA^{Phe}$ gene. Initially, proteins in the form of 42S particles were used and it was considered necessary to disrupt the 42S RNP particle by treatment with 8M urea, followed by renaturation of protein in the presence of DNA, by dialysing out the urea. However, straightforward mixing of 42S RNP particles with DNA in buffered salt solution was found to be equally effective in forming protein-DNA complexes (fig.37). Reconstitution in the presence of RNAse resulted in more of the DNA being bound by proteins (fig.37A), presumably because protein is displaced from the RNP particle on RNA digestion. Using the mixing procedure with RNase, the affect of NaCl

Fig.37. Gel retention of tRNA gene sequences by 42S particles. In each reaction 5ug of 42S particles were mixed with 2ng of 5' end-labelled DNA. The basic reaction conditions is 100mM NaCl, 10mMTris-HCl, pH7.4, 2mM $MgCl_2$ plus 1ug of pBR322 and 0.1 units of RNase A, T1 and T2 in a reaction volume of 20ul. (A). (-) 42S particles plus the 269bp EcoRI/HindIII insert of pJ52 without RNAse present during the reaction; (+) 42S particles plus 0.1 units of RNAse A, T1 and T2, with either the 269bp EcoRI/HindIII insert of pJ52 or a 232bp fragment from pUC9. (B) The effect of salt concentration. 42S particles were incubated with the 269bp EcoRI/HindIII insert of pJ52 in the presence of RNAse with the different NaCl concentrations indicated (Track M - no protein added to the reaction).


A
+100mM NaCl
RNase
M
-
+
+
M
tDNA
pUC


B
+RNase
NaCl
M
100mM
50mM
100mM
150mM
200mM
500mM
tDNA

concentration on formation was tested. As fig.37B shows increasing NaCl concentration above a certain level decreases the ability of the proteins to bind the DNA. The optimal concentration for stable complex formation is about 100mM NaCl. These results are comparable with the binding of factor Υ to the yeast $tRNA_3^{Glu}$ gene, where optimal binding is at 135mM KCl (41). At optimal NaCl concentration for 42S RNP particle protein binding, a 232bp end-labelled fragment from pUC9 was unable to bind to 42S RNA particle proteins as a specific complex (fig.37A).

With this method of analysis two complexes may be observed in the gel. Fig.38 shows the effect of increasing protein concentration in the assay. As the amount of free DNA decreases in this assay the amount of complexII increases proportionally. However, complexI stays at a relatively constant level. ComplexI could be an intermediate in the formation of complexII as this complex increases in amount in competition assays (see below).

Further analysis of protein-DNA interaction was carried out by competing unlabelled pJ52 with the labelled $tRNA_1^{Met}$ gene. Fig.39 shows that when pJ52 is added before the ^{32}P-labelled $tRNA_1^{Met}$ gene, then the formation of a specific labelled complex is prevented. However, when added together, competition occurs resulting in a decreased amount of labelled protein-DNA complexII and also a change in the apparent size of this complexI. This change may result from a sub-maximal binding of 42S RNP particle proteins to the labelled DNA.

Fig.38. The effect of protein concentration on DNA binding. 2ng of 5' end labelled NsiI fragment of pXlt81 containing the $tRNA_1^{Met}$ gene was incubated with different amounts of 42S particles. in the presence of RNAse and 100mM NaCl. (lane 1), no 42S particles; (lane 2), 0.125µg of 42S particles; (lane 3), 0.25µg of 42S particles; (lane 4), 0.5µg of 42S particles; (lane 5), 1.0µg of 42S particles; (lane 6), 2.0µg of 42S particles.

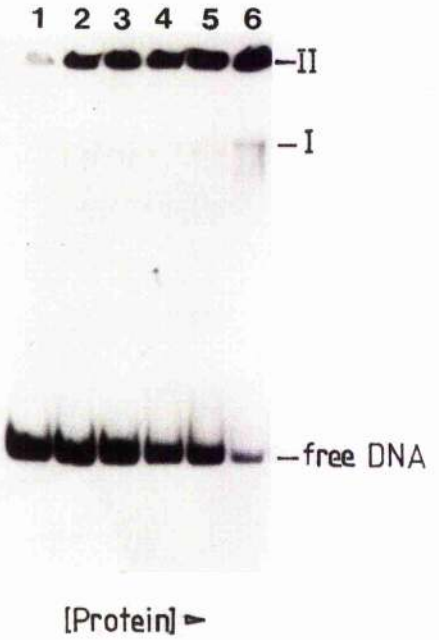
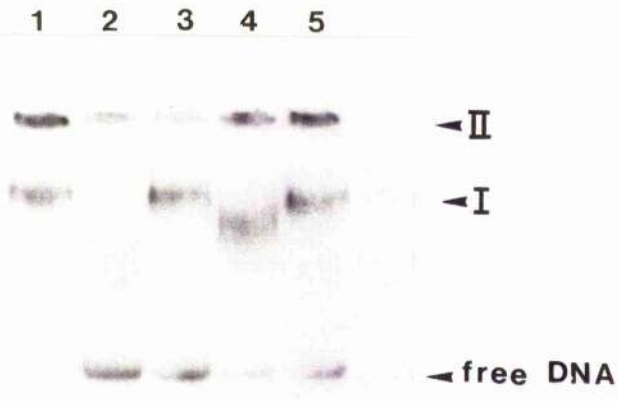

1 2 3 4 5 6
–II
–I
–free DNA
[Protein] ►

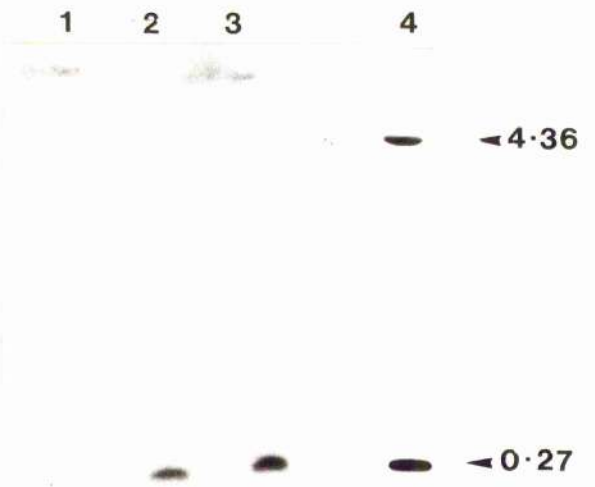
Fig.39. Competition of tRNA gene sequences for 42S particle proteins. Reactions conditions were as in Fig.35 (+) except the the NsiI fragment of pXlt81 was used as the labelled DNA probe. lane 1. 0.25µg of unlabelled pJ52 was added 10 mins before the labelled DNA was added. lane 2. as lane 1 except 1ug of unlabelled pJ52 was added. lane 3. 0.25µg of unlabelled pJ52 was added simultaneously as the labelled DNA. lane 4. as lane 3. except 1µg of unlabelled pJ52 was used. lane 5. no competitor added.


1
2
3
4
5
II
I
free DNA

2:15 Immunopreciption of protein-tRNA^{Phe} gene complexes with anti-42S particle protein antibodies directed against the proteins of 42S particles.

Selective immunoprecipitation of TFIIIA-5S RNA gene complexes has been used to locate bases in the 5S RNA gene sequence important for interaction with TFIIIA (40). Selective precipition by antibody-bound protein-A Sepharose beads was used to confirm the nitrocellulose filter binding data shown above (section 2:14). 42S particles were added to ^{32}P-labelled tRNA^{Phe} gene containing fragments and protein-DNA complexes were allowed to form. Following protein-DNA complex formation, anti-P48 or anti-P43, was added and after incubation, immunoprecipitates were collected and any labelled DNA that they contained was analysed by gel electrophoresis /autoradiography. It was found that anti-P43 and anti-P48 were equally effective in specifically precipitating the tRNA gene fragment (fig.40). This result is different from that obtained with DNA binding to protein immobilized on nitrocellulose (fig.35) where only P43, and not P48, bound the tRNA. gene fragment. A possible explanation is that P43 is the only protein that interacts directly with the gene but that P48 has a strong affinity for P43 or for the P43/DNA complex. In fact a strong interaction of radioactively labelled P48 with P43 immobilised on nitrocellulose is found (A.D.Lomas pers. comm.).

Fig.40. Immunoprecipition of 42S particle protein/DNA complexes by anti-P48 and anti-P43. 5' end-labelled EcoRI/HindIII cut pJ52 was mixed with 5ug of 42S or 7S particles under the same conditions as described in Fig.35, (+). After 30 mins, antibody linked protein-A sepharose was added and agitated for 1hr. The beads were removed and washed six times. The DNA was released from the complex by proteasing and analysed on an 8% polyacrylamide gel. lane 1. 7S particles immunoprecipitated with anti-P40 protein-A sepharose. lane 2. 42S particles immunoprecipitated with anti-P43 protein-A sepharose. lane 3. 42S particles immunoprecipitated with anti-P48 protein-A sepharose. lane 4. EcoRI/HindIII digest of pJ52 (sizes in Kb).


1
2
3
4
4·36
0·27

2:16 Protection of the 269bp DNA fragment containing $tRNA^{Phe}$ gene by 42S particles

One approach to localize protein-DNA interactions is by the method of DNAse footprinting. Briefly, this involves the formation of protein-DNA complexes, using ^{32}P end-labelled DNA then digestion with sufficient DNAse1 to cut on average once per DNA molecule. The products of the reaction are separated on a DNA sequencing gel and a ladder of bands is obtained corresponding to each base in the DNA fragment. All bases will be observed except those protected from digestion by steric hinderance of DNase by the bound protein.

This approach has permitted localization of DNA sequences involved in transcription factor binding to 5S RNA gene (97), yeast $tRNA_3^{Glu}$ gene (41) and yeast $tRNA_3^{Leu}$ gene (157).

The $tRNA^{Phe}$ gene used here does not contain any intervening sequences and should be efficient in binding transcription factors (160,181). It has been reported that yeast $tRNA_3^{Glu}$ gene and yeast $tRNA_3^{Leu}$ gene bind transcription factor(s) which are able to protect both the 'A' and 'B' box regions of the DNA (41,157). In the former case, the entire gene is protected.

Protein-DNA complexes for footprint analysis were reconstituted using RNAse treated 42S particles, by either dialysis in the presence of 8M urea or by the addition of all components to an Eppendorf tube. Both procedures gave similar, although slightly different quantitative results.

For reactions performed in Eppendorf tubes, significant amounts of the whole 269bp fragment are protected from digestion with DNAse1 This protection was afforded at 20 times the concentration required to cleave the unprotected DNA on average once per DNA molecule (fig41) A ladder of fragments is seen in these reactions but the increase in smaller fragments at the expense of large fragments, whilst leaving the amount of whole fragment quantitively unchanged when the amount of DNAse is increased, is indicative of a proportion of fragments being unbound by any protein.

Reconstitution of a protein-DNA complex by dialysis leaves greater than 90% of the 269bp fragment protected form DNAse digestion with a DNAse 1 concentration at 5µg/ml and digestion for 5 mins. (fig.41, lane1). However, there is some indication of 3' and 5' trimming. An approximate value for the extent of protection is -25 to +145

As demonstrated in the previous section, protein-RNA complexes may be reconstituted in vitro using gel purified 42S proteins and gel purified 5S RNA and tRNA (fig.2). This approach was used in protection studies by dialysing out the SDS present in the elutions of isolated proteins. However, following this treatment the purified proteins are not able, either singly or together, to afford any protection to the gene or its flanking sequences (fig.42). The possibilty is that P48 and P43 which are able to recognize the secondary structures of 5S RNA and tRNA (60,61, J.Sommerville pers. comm.) are not able to recognize specific base sequences either because of gross denaturation of the DNA binding site or the presence of sufficient residual SDS to prevent binding.

Fig.41. DNase1 footprinting of 42S particle protein-DNA complexes. The 269bp insert of pJ52 containing the $tRNA^{Phe}$ gene was labelled at the 5' end of the coding strand. Protein-DNA complexes were formed between 42S particle proteins and labelled DNA as decribed in Fig.37, (+) and digested with DNase1 at varying concentrations and times. The digestion was stopped and the DNA fragments were phenol extracted and prepared for electrophoresis on an 8% sequencing gel.

lane 1. 50ng DNase1, 120sec digestion but protein and DNA were allowed to associate in the presence of 8M urea then dialysed against reconstitution buffer.

lane 2. 5ng DNase1, 30sec digestion

lane 3. 10ng DNase1, 30sec digestion.

lane 4. 10ng DNase1, 60sec digestion.

lane 5. 50ng DNase1, 30sec digestion.

lane 6. 50Ng DNase1, 60sec digestion.

lane 7. 50ng DNase1, 120sec digestion.

lane 8. 5ng DNase1, 30sec digestion.

lane 9. 5ng DNAse1, 30sec digestion in the absence of protein. Protection of DNA is not due to binding of RNase as shown in lane 9.

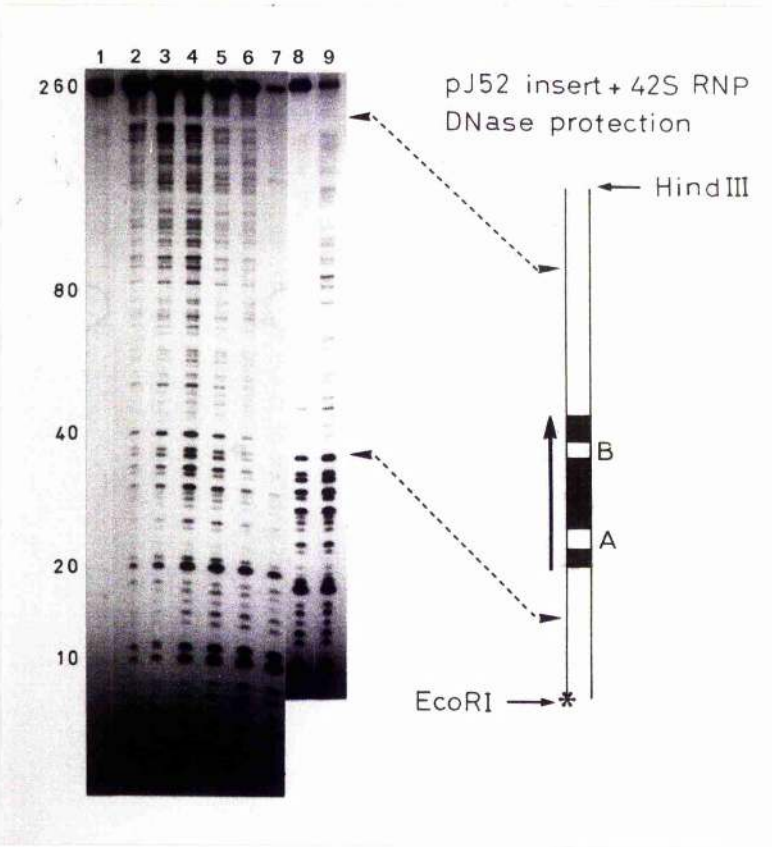

1 2 3 4 5 6 7 8 9
260
80
40
20
10
pJ52 insert + 42S RNP
DNase protection
HindIII
B
A
EcoRI
*

Fig.42. DNase1 footprinting of 42S particle protein-DNA complexes. The 269bp insert of pJ52 containing the $tRNA^{Phe}$ gene labelled at the 5' end of the coding strand was reconstituted with isolated P43 (lane 1) or P43 plus P48 (lane 2) in 8M urea followed by dialysis against reconstitution buffer minus urea. Digestion was then carried out with 100ng of DNase1 for 30sec. lane 3 is digestion in the absence of protein.


1 2 3

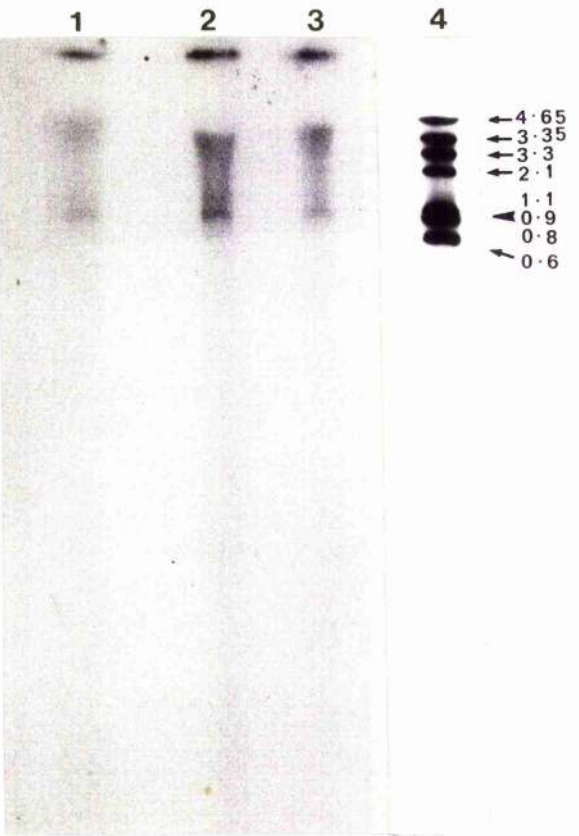
The 42S particles themselves, without RNAse treatment afford only a small amount of protection to $tRNA^{Phe}$ gene indicating that the 42S particles are fairly stable complexes and do not exchange RNA for DNA at a very fast rate (data not shown).

2:17 specific interaction of rDNA with 42S particle proteins.

As noted earlier, rDNA containing plasmid interacts with P43. This interaction was investigated further by using the immunoprecipitation technique.

Reconstitution of protein-DNA complexes was attempted with both 42S RNP and 7S RNP particles and 5' end labelled BamH1/EcoR1 digested pXlor101. After complex formation, anti-P43 was found to immunoprecipitate two fragments of 1.1Kb and 3.6Kb (fig.43). These fragments contain the gene promotor and the multiple 60/81bp repeats of the non-transcribed intergenic spacer. The multiple repeats are related in sequence to part of the gene promotor (33,171,182,183). Immunoprecipipition with this antibody bound to protein-A Sepharose is significantly greater than anti-P40 or anti-P48 bound protein-A sepharose. No immunoprecipitation can be seen using anti-P43 or anti-P40 with mixture of 7S RNP particles.

Fig.43. Immunoprecipitation of 42S particle protein-rDNA complexes by anti-P43. 5' end-labelled BamHI/EcoRI cut pXlor101 (lane 4) was mixed with 5ug of 42S or 7S particles as described in Fig.35, (+) After 30mins, antibody linked protein-A sepharose was added and agitated for 1hr. The sepharose was removed and washed six times. The DNA was released from the complex by proteasing and analysed on an 8% polyacrylamide gel. lane 1. 7S particles immunoprecipitated with anti-P40 protein-A sepharose. lane 2. 42S particles immunoprecipated with anti-P43 protein-A sepharose. lane 3. 42S particles immunoprecipitated with anti-P48 protein-A sepharose. lane 4. BamHI/EcoRI digest of pXlor101 (sizes of fragments in Kb).


1
2
3
4
4·65
3·35
3·3
2·1
1·1
0·9
0·8
0·6

DISCUSSION

The interaction of 42S RNP particle proteins with tRNA genes was explored in five types of experiments. The initial observation was the reduction of tRNA synthesis on injecting anti-P43 IgG simultaneously with plasmid pXlt81 containing containing $tRNA_1^{Met}$ gene. This suggests that P43 normally has a positive effect on tRNA transcription (Fig.34B) and a working hypothesis is that P43 exerts this effect through direct binding to regulatory regions in tRNA genes. The interaction of antibodies with P43 might then inhibit synthesis through interferring with its DNA-binding potential. However, the effect of the antibody is not complete inhibition of synthesis. One explanation for only partial inhibition is that the time it takes to form anti-P43-P43 complexes is greater than the time if takes to form P43-tRNA gene complexes. Indeed, once a P43-tRNA gene complex has formed, it is likely to be stable and unaffected in its activity by anti-P43. This is shown in Fig.34B where anti-P43 was injected with 5S DNA and no inhibition of endogenous tRNA synthesis was seen. The inhibition of tRNA synthesis is also observed when anti-P48 IgG is coinjected along with pXlt81. The reduction in synthesis by both antibodies is significant (3-4 fold) when compared to the effect of pre-immune IgG and anti-P40 IgG.

RNA polymerase III reconstituted systems require at least two protein fractions after ion-exchange chromatography (20,34,184,185, 186). Stable pre-initiation complexes are able to form between gene and accessory factors (90,160,187,188). Factor C is able to bind stably to any tRNA gene, whereas factor B does not (160). The

inhibition of tRNA synthesis from pXlt81 by anti-P43 and anti-P48, does not tell us whether P43 or P48 interact with the DNA directly or as an auxillary factor through the association of another unrelated protein binding to tRNA genes first. However, the assay of complex formation by polyacrylamide gel electrophoresis shows that sucrose gradient fractions containing 42S particles are able to displace the 269bp DNA fragment containing $tRNA^{Phe}$ gene to the top of the acrylamide gel. The large displacement suggests that a large complex of proteins have bound to the DNA fragment (177,178), (Fig.37A). Intermediate complexes in the formation of this large complex (complexII) may also occur (complexI, Fig.38). This binding was sensitive to the salt concentration present during binding (Fig.37B). The optimal concentration was 100mM NaCl. This is comparable to factor τ binding to $tRNA_3^{Glu}$ gene (41). Under the conditions employed for binding (100mM NaCl), 42S particles stability is reduced (57,64,66), but treatment of the 42S particles with RNAse was necessary to acheive total displacement (Fig.37A). The conclusion from this experment is that 42S particles are able to interact directly with the DNA and that the translocation of protein is dependant on the destabilistion of the particles and promoted by removal of the RNA.

P43 and P48 both regenerate sufficient secondary and teriary structure to bind 5S RNA (P43 and P48) and tRNA (P48, figs.20 and 21) on nitrocellulose filters. This technique, originally developed to detect DNA-binding proteins (84), was applied here to detect the ability of the 42S particle proteins to bind DNA fragments containing tRNA genes. Initial studies with nick-translated pXlt81 indicated

that only P43 was able to bind the $tRNA_1^{Met}$ gene (Fig.35). This was confirmed using the 269bp insert of pJ52 containing the $tRNA^{Phe}$ gene (Fig.36). Binding was carried out in the presence of cold E.coli total DNA and P43 did not bind ^{32}P-labelled pBR322, indicating the specificity of the binding of P43 for the 269bp insert.

Results of other workers suggest the region of DNA containing the 'B' box to be most important in binding transcription factors (157,189), These factors either bind exclusively to the 'B' box or bind to this region with greater rapidity and affinity.

Fig.36 shows the ability of P43 to bind DNA fragments containing either the 'A' box sequence or the 'B' box sequence. The amount of each fragment bound by P43 is similar, so little information can be gained from this experiment with respect to the relative affinity of the fragments for P43, or indeed which region of DNA is responsible for the initial contact. However,it is concluded that P43 can bind to sequences in both portions of the DNA. The contacts may be formed through interaction with intragenic sequences or with 5' or 3' flanking sequences. If binding occurs through both the 'A' and 'B' boxes, the results seen here would be consistent with the ability of other tRNA transcription factors to bind both 'A' and 'B' boxes (41,157,160) and the ability of the 'A' box or 'B' box to promote transcription independently of each other (188).

Studies with the yeast tRNA transcription system (160) have shown that a factor C is required for the formation of a stable pre-initiation complex with specfic tRNA genes (eg. ARG and SER), but is highly unstable in forming complexes with other tRNA genes

(LEU_3 and TYR). This instability was due, in the case of $tRNA^{Leu}_3$, to the length of intervening sequence between the 'A' and 'B' boxes. In the case of $tRNA^{tyr}$, instability was due to the slight deviation from the optimal 'A' and 'B' box sequences. These results show both 'A' and 'B' box sequences play a role in binding of factor C and is dependent upon the the spatial relationship between the two boxes is important.

The formation of a stable complex with tRNA. in the yeast system (160), involving factor C and fraction B, highlights the cooperation between these factors to form a pre-initiation complex. Both anti-P48 and anti-P43 are able to immunoprecipitate the 269bp fragment containing $tRNA^{Phe}$ gene . One possible interpretation of this, together with the results in figure.34B. is a cooperation in binding between P43 and P48. P43 is the only protein of the two that binds the $tRNA^{Phe}$ gene containing fragment by the nitrocellulose binding assay. This would suggest anti-P48 can inhibit tRNA synthesis by preventing a stable complex formation between P43 and P48.

Interaction of transcription factors with DNA can be visualized directly using the techinque of DNAse footprinting (35). Transcriptionally competent cell extracts from human cells (185) or yeast (41,157,190) were found by DNAse footprinting to protect strongly the intragenic control regions of tRNA genes. In these experiments, protection boundaries extended into the 3' and 5' flanking sequences by up to 10bp. Protection of most genes spanned the entire length of the gene into these flanking sequences. However, in the case of $tRNA^{Leu}_3$ gene, which contains an intron of

31bp, protection of the region of DNA containing the intervening sequence does not occur (157).

In the experiments described here, the 269bp tRNA^{Phe} gene containing fragment is protected entirely from digestion with DNAse 1 by 42S particles, when reconstitution is carried out in the presence of RNAse (Fig.41). This reconstitution can be achevied by dialysis through 8M urea to ensure that the 42S particle proteins remain soluble after removal of the RNA. Protection extends from -67 to +192. With extensive digestion by DNAse 1, 5' and 3' trimming becomes evident but with complete protection extending from approximately -25 to +145. This region still encompasses the entire region of the tRNA. gene. Accessibility of DNAse 1 to the DNA when bound to 42S particle proteins may be the explanation for extensive protection. It is not merely the presence of 42S particles (or contaminant, minor proteins in this fraction) causing the inhibition of DNAse 1 because the enzyme extensively digests the fragment in the presence of 42S particles without RNAse (data not shown).

Transcriptional activity of mutated tRNA genes shows a large degree of variability in both heterologous and homologous systems. Removal of sequences at the 3' end of the tRNA^{Leu} gene of X.laevis completely abolished transcription (155). This and other results suggested sequences encoding the conserved T arm (+52 to +62) are essential for transcription (21,191). However, a modified tRNA^{Arg} gene of Drosophila containing only the 'A' box sequences and a tRNA^{Arg} gene lacking the 'A' box sequences were active in Xenopus extracts (188).

In addition to the intragenic promoter, upstream sequences are required by some tRNA genes for efficient transcription (136,193-195). An absolute requirement for these 5' flanking sequences is demonstrated with 5S RNA genes and tRNA genes of Bombyx mori (192,196,197). However, Xenopus 5S RNA genes do not require flanking sequences, for their transcription in vitro. It has been demonstrated with $tRNA_2^{Ala}$ gene of B.mori, that the region from -13 to -50bp beyond the end of the tRNA gene (+149) is required for efficient transcriptional activity and the 3' end of this gene (+20 to +146) influence transcription factor binding (198). The results of the above footprinting experiments are consistent with the findings of Wilson et al. (198) and also consistent with the results of the gel retention assay with the Xenopus $tRNA^{Phe}$ gene or $tRNA_1^{Met}$ gene (Fig.38) indicating the formation of a large protein complex on these genes. Also both coding and non-coding strands are protected from DNAse 1 activity and this also is true of the protection of the $tRNA_3^{Glu}$ gene of yeast (41).

Reconstitution of protein-DNA complexes with gel purified 42S particle proteins was unsuccessful, using either P48 or P43 or both together (Fig.42). This failure to interact with the DNA could be the result of the presence of residual SDS bound to the protein. Although in the nitrocellulose filter binding assay, P43 is initially complexed with SDS, transfer of the proteins to nitrocellulose in an electric field probably dissociates SDS and protein. This should then allow the protein to interact with DNA.

Although it has been reported that 42S particle proteins do not bind 5S DNA (62), it is demonstrated in Fig.35 that P48 immobilized on nitrocellulose is able to bind the plasmid pXlo8 containing the X.laevis oocyte-type 5S DNA. Binding also occurred to P40 (TFIIIA) under the same conditions which favoured only tRNA gene binding to P43. The involvement of P48 in 5S RNA transcription is demonstrated by co-injecting pXlo8 and anti-P48 IgG. Synthesis of 5S RNA is reduced 3-4 fold by anti-P48 or anti-P40, but not by anti-P43. These results suggest the interaction of P48 could be either by direct binding to the DNA or by preventing a P48-5S RNA interaction, which in turn inhibits synthesis of 5S RNA by feedback inhibition (62) in the same way as excess 5S RNA is bound by TFIIIA.

Futher clarification of the binding of 5S DNA by P48 is necessary to draw any conclusions about its possibile role in 5S RNA gene expression. It is known that oocyte-type 5S DNA has affinity for TFIIIA of only 1/4 of that of the somatic-type 5S DNA (121). This is due to differences in the sequences of the internal control region at postions +53, +55 and +79. There is evidence to suggest that a transcriptional complex consisting of TFIIIA, B and C is removed by a replication fork proceeding through a 5S RNA gene (A.P.Wolffe, pers comm.). 5S RNA gene control could then be mediated through this 4 fold greater affinity for somatic type 5S RNA genes. During development, at each round of DNA replication, somatic 5S RNA genes bind an ever diminishing amount of TFIIIA. Hence it would not require many rounds of replication to inactivate the oocyte-type 5S RNA genes.

RNA polymerase 1 requires as many as four transcription factors for the correct initiation of transcription from ribosomal genes located at the nucleolus (42). At least some of the transcripton factors for ribosomal RNA synthesis are species-specific (11). One of these factors (TFIA) is cell cycle regulated and possibily interacts directly with RNA polymerase 1 (199). TFIB on the otherhand is present in both growing and growth- aressted cells, forming a stable pre-initiation complex, responsible for the selectivity of rDNA transcription (199).

Transcription activity of rRNA genes in Xenopus is influenced by 'enhancer' sequences in the spacer DNA which share homology with the Xenopus rDNA promotor (15). These sequences, 60 or 81bp elements, confer 20-fold dominance in transcription activity over genes lacking these enchancers.

The results shown in Fig.35 show the ability of P43 to bind plasmid pXlor101 which contains rDNA sequences. This binding was investigated further using the immunoprecipitiion technique. The results are shown in Fig.43. It appears that P43 is able to bind selectively to two fragments of 3.6Kb and 1.1Kb. These fragments both contain spacer DNA sequences (Fig.33) which will contain the duplicated gene promotors.

Consistent with these observations is the data presented in Fig.31. and immunoflorescence studies (J. Coxon pers.comm.). These experiments show the presence of radiolabelled proteins at the nucleoli after microinjection into stage 3 oocytes of labelled 42S particles and immunofluorescent staining of nucleoli with anti-P43

but not with anti-P48 or anti-P40.

Within the 60/81bp repeat is a 42bp region which has 80-90% homology with the -72 to -114 region of the gene promotor (200,201). DNAse 1 footprinting suggests that the transcription factor(s) for rRNA genes in Xenopus bind and protect this region (171). It remains to be determined whether P43 binds and protects this region of the spacer DNA.

Fractionation of mouse cellular extracts over DEAE-Sephadex results in the co-purification of RNA polymerase 1 together with the cell cycle regulated transcription factor (199). Fractionation of a X.laevis oocyte extract over DEAE-Sephadex results in the appearance of P43 in fractions containing RNA polymerase 1 (data not shown). Although P43 co-elutes with RNA polymerase 1 further purification and functional tests for P43 influence on RNA polymerase 1 activity is required to establish unequivocally transcriptional factor activity of P43 on rRNA synthesis.

Co-ordinate expression of ribosomal components involves the co-ordination of the synthesis of 5S RNA, 18S, 5.8S and 28S RNA and genes coding for ribosomal proteins. Although in Xenopus oocytes there appears to be discoordinate synthesis of 5S RNA from other ribosomal components, the data present in sections 1 and 2 may be reconciled with this observation.

One possibilty is that P43 plays a central role in bringing all these activities together. Firstly its effect on the regulation of tRNA transcription through protein-DNA interaction (figs.34B, 38 and 40) assisted perhaps by P48 (figs.34B and 40). Secondly , its

involvement in the transportation of 5S RNA to the nucleolus for incorporation into ribosomes (Fig.32). Thirdly, the use of its possible cleavage product of 17kD to become an integral part of the ribosome structure (Fig.25).

A model may be conceived whereby the amount of P43 could regulate the entire amount of protein translational machinary. From this point of view, how the production of P43 itself is regulated would be of extreme importance. For instance if the gene struture for P43 were similar to the structure of the gene for TFIIIA, where a sequence identical to the internal control region of 5S RNA genes is part of the gene for TFIIIA (59), autogenous regulation through the binding of the protein product to a structure in its own gene or gene transcript, would be a distinct possibilty.

Summary

In summary, it is shown that the 42S particle is not merely a simple storage vessel for 5S RNA and tRNA during oogensis, but has a more dynamic role in controlling the supply of components to the protein translational machinary.

Firstly heterogeneity in composition of the 42S particle was observed, ranging from 42S particles whose only major protein is P48 at early stage 1 to particles containing variable amounts of P43 in relation to in the relative amount of P48 as oogensis progresses at stage 2-3. The decrease in P48 is in part due to cleavage of this protein to a new product of 33000 which can persist with 5S RNA until the end of oogenesis as a 7S RNP. Heterogeneity within the 42S particle can also be explained in terms of the ability of P48 to bind either 5S RNA (1 molecule) or tRNA (up to 3 molecules). Although P43 can form a stable complex with 5S RNA, and appears to be active in the utilization of 5S RNA in ribosome formation, its interaction with tRNA is less effective and it may have no role in vivo. Heterogeneity can derive, therefore, from different combinations of these complexes.

Although immunological analysis showed P48, P43 and P40 (TFIIIA) to be structually distinct, cyanogen bromide peptide cleavage was carried out to confirm this. These data together with amino acid analysis firmly establish the three proteins P48, P43 and P40 to be seperate gene products.

The detection of a smaller cross-reacting protein in the 60S subunit of Xenopus ribosomes with the antibody anti-P43 suggested the involvement of P43 in ribosome synthesis. Labelled 42S particles (^{32}P-5S RNA or ^{14}C-protein labelled) injected into Xenopus oocytes resulted in the appearence of a proportion of the label in ribosomes. A protein of the same molecular weight prepared from somatic ribosomes also cross-reacted with this antibody. The use of ^{35}S-cysteine-labelled 42S particles in cytological studies has shown uptake of label into nucleoli and immunofluorescence studies have shown that anti-P43 reacts with nucleoli. These observations are consistent with the view that P43 accompanies 5S RNA to the nucleolus and may be cleaved to P17 before incorporation into the ribosome structure.

The role of 42S particle proteins in transcription of 5S RNA and tRNA was investigated. P48 was shown by nitrocellulose-binding assay to interact with 5S RNA genes and anti-P48 was shown to inhibit transcription of 5S RNA in vivo. Anti-P48 in microinjection experiments also inhibited tRNA transcription in vivo, whereas anti-P40 could not do so. In an immunoprecipitation assay anti-P48 could also bring down a P48/tRNA gene complex.

The role of P43 in transcription was shown in the nitrocellulose-binding assay to bind tRNA genes. An immunoprecipitation technique also confirmed this result. In vivo, anti-P43 was able to inhibit tRNA synthesis but not 5S RNA synthesis. DNAse 1 footprinting studies show that a large region of the tRNA^{Pro} gene is protected by proteins from 42S particles.

Finally, P43 may have a role in ribosomal RNA synthesis as suggested in the nitrocellulose-binding assay in which DNA containing a rRNA gene transcription unit was able to bind P43. Also in an immunoprecipitation assay P43 was able to immunoprecipate P43/DNA complexes, the DNA being two fragments of 3.6Kb and 1.1Kb of an EcoR1/BamH1 digest of pXlor101. These fragments of DNA contain the 60/81bp repeats which share homology to the gene promotor which resides in the 3.6Kb fragment.

All these results taken together suggest a central role for 42S particle proteins, in particlular P43 in co-ordinating the synthesis of the protein translational machinary of the oocyte.

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